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Studies on the 'Fermentation' of Ceylon Tea

4. ESTIMATION OF THE OXIDIZING ENZYME ACTIVITY

By H. B. SREERANGACHAR, *Tea Research Institute of Ceylon, Talawakelle*

(*Received* 8 *July* 1943)

In our previous studies [Lamb & Sreerangachar, 1940*a*, *b*] the oxidizing activity of the tea-leaf enzyme was measured by following the changes in the tannin content of an enzyme-substrate reaction mixture by the iodine titration method. In stressing the limitations of this method it was stated that it did not afford any indication of complex changes such as condensation, and there existed a possibility that the results were subject to an error due to these causes.

The oxidizing enzyme in tea leaf is a polyphenol oxidase and ascorbic acid is only indirectly oxidized in the presence of a suitable polyphenol. The function of the polyphenol in this reaction is that of an oxygen carrier, and its permanent oxidation becomes noticeable only after the complete oxidation of all ascorbic acid. The *o*-quinones are continually removed from the sphere of reaction and therefore their condensation cannot occur as long as there is an excess of ascorbic acid in the system. The ascorbic acid reaction thus provides the basis of a more reliable method for measuring enzyme activity in the tea-leaf enzyme preparation. Ezell & Gerhardt [1940] have likewise employed ascorbic acid as a substrate in the determination of oxidase activity in fruits and vegetables.

EXPERIMENTAL

Enzyme preparation and properties. The enzyme preparation employed in this investigation was obtained by grinding tea leaf with sand under acetone, washing until free of all colouring constituents and rapidly drying under vacuum. This acetone-prepared leaf powder was all but polyphenol-free and exerted only a very weak oxidizing action on pure *l*-ascorbic acid. Gradual removal of the associated polyphenols from this tissue powder rendered the enzyme progressively inactive towards ascorbic acid. Thus on extraction with water and exhaustive washing, a large fraction of the residual tannin was removed and the ascorbic acid oxidation activity fell markedly. Aeration for 2–3 hr. of a suspension in water of the extracted enzyme powder did not remove any further quantities of polyphenol; grinding the extracted powder with sand and water, filtering and washing resulted in the material still giving a positive ferric chloride reaction for tannin substrates. However, when the ground mass was treated with an excess of a dilute solution of ascorbic acid it was found possible to extract completely the last traces of polyphenol. The ascorbic acid oxidation activity of this final preparation is almost negligible. Table 1 gives results showing both the polyphenol oxidase activity on a prepared tea-tannin substrate solution measured iodometrically and the ascorbic acid oxidation activity estimated by the use of the 2:6-dichlorophenol-indophenol method at the above different stages of extraction. The reactions were all carried out in presence of McIlvaine's buffer at pH 5·4.

Table I. *Effect of polyphenol removal on enzyme activity*

Extraction stage	Activity/0·1 g. enzyme powder: Polyphenol oxidase activity (4 hr.) (ml. N/20 $Na_2S_2O_3$)	Activity/0·1 g. enzyme powder: Ascorbic acid oxidation (5 min.) (mg. ascorbic acid)
1. Unextracted powder	10·5	0·29
2. After extraction with McIlvaine's buffer at pH 7·0 and complete washing	12·8	0·09
3. After aeration	12·0	0·07
4. After grinding with sand and water	—	0·06
5. After treatment with ascorbic acid	18·4	0·002

These results indicate that the enzyme in question is not ascorbic acid oxidase but that it is polyphenol oxidase, of which the activity increases on partial purification. Since the complete removal of all tissue-bound polyphenols renders the enzyme practically inactive towards ascorbic acid there can be no reasonable doubt that the ascorbic acid oxidation activity of the unextracted powder was due to an indirect action of a polyphenol-polyphenol oxidase system.

The ascorbic acid oxidation activity exhibited by the enzyme in the presence of polyphenol was, however, found to vary according to the amount of polyphenol present. It became, therefore, necessary to find out the optimum quantity of polyphenol that would give the maximum enzyme activity. Two polyphenols, catechol and amorphous tea tannin, were so studied and Table 2 shows the effect of their addition on ascorbic acid oxidation by the enzyme. The reaction mixture consisted of 5 ml. 0·1% ascorbic acid solution, 4 ml. distilled water, 1 ml. catechol or tea-tannin solution containing various amounts of polyphenol, and 20 mg. acetone-prepared enzyme powder. The mixture was aerated for 1 hr., after which it was filtered and 5 ml. titrated with a standard solution of 2:6-dichlorophenol-indophenol. Neither 100 mg. catechol nor 20 mg. tea tannin, the maximum amounts of polyphenols employed in this experiment, gave any blank 2:6-dye titre when dissolved in 5 ml. A control experiment in which the polyphenol

solution was replaced by 20% solution of trichloroacetic acid was also run at the same time, and the enzyme activity obtained by the difference between the control and experimental values.

Table 2. *Effect of addition of polyphenols on ascorbic acid oxidation activity*

Catechol			Tea tannin		
	Enzyme activity (mg. ascorbic acid)			Enzyme activity (mg. ascorbic acid)	
mg. added	(*a*)*	(*b*)*	mg. added	(*a*)*	(*b*)*
0	0·27	0·98	0	0·80	0·98
0·5	0·82	—	0·5	1·16	—
1·0	0·96	—	1·0	1·20	—
2·0	0·92	—	2·0	1·59	—
3·0	0·98	—	4·0	1·63	—
4·0	1·06	—	5·0	—	3·77
5·0	1·33	—	10·0	—	4·06
10·0	1·53	—	20·0	—	5·02
15·0	1·94	—	40·0	—	4·53
20·0	2·10	—	—	—	—
25·0	2·22	2·90	—	—	—
50·0	—	2·04	—	—	—
100·0	—	1·22	—	—	—

* (*a*) and (*b*) represent two separate enzyme preparations.

The results (Table 2) show that in a total volume (10 ml.) of the reaction mixture either 25 mg. catechol or 20 mg. tea tannin gave optimum values for ascorbic acid oxidation activity, which is higher in the case of tea tannin than catechol. But as the preparation of tea tannin in pure state is tedious and its identity still uncertain it was thought desirable to employ only the more definite compound, catechol. Instead of using catechol and ascorbic acid solutions separately it is convenient to prepare a mixed substrate solution containing 0·1% ascorbic acid and 0·5% catechol. 5 ml. of this solution, containing 5 mg. ascorbic acid and 25 mg. catechol, are usually employed in each reaction.

Optimum pH *and temperature.* The optimum conditions for ascorbic acid oxidation by the tea-leaf enzyme system are shown in Table 3. McIlvaine's buffer at various pH values was employed and the reactions for determination of optimum temperature were carried out in a thermostat at pH 5·4. There was slight autoxidation of ascorbic acid at pH > 6·0 and the values were corrected for such autoxidation errors.

Table 3. *Optimum* pH *and temperature*

	Enzyme activity (mg. ascorbic acid)			Enzyme activity (mg. ascorbic acid)	
pH	Mixed enzyme	Insoluble enzyme	Temp. (°C.)	Mixed enzyme	Insoluble enzyme
4·0	2·34	2·00	16	1·14	1·22
4·6	2·48	1·80	21	1·59	1·47
5·0	2·85	—	27	1·79	1·92
5·4	2·73	2·53	32	1·26	1·71
6·0	1·82	1·84	38	1·06	1·29
7·0	1·49	1·13			
8·0	1·36	1·18			

The mixed and insoluble enzymes were derived from different samples of leaf. The optimum pH for the mixed and the insoluble enzymes is 5·0 and 5·4 respectively, while both enzymes have the same optimum temperature, 27°.

Relation between soluble, insoluble and mixed enzymes. In earlier investigations it was shown that the activity of the unextracted powder was not equal to but always less than the sum of the activities of the extract and the residue. When this question was reinvestigated by the ascorbic acid method, it was observed that the activity of the unextracted powder consisted of those due to the soluble and insoluble enzymes. To a mixture of 5 ml. of ascorbic acid-catechol substrate and 5 ml. McIlvaine's buffer at pH 5·4 the enzyme was added in the following forms: (1) unextracted powder (20 mg.), (2) insoluble enzyme from 20 mg. unextracted powder, and (3) enzyme extract (2 ml.) derived from 20 mg. unextracted powder. The reactions were carried out separately. At the end of 1 hr., the enzyme activities were estimated by titration of 5 ml. filtrates in the case of solid enzymes and 6 ml. in the case of the enzyme extract. The results are shown in Table 4.

Table 4. *Activities of soluble, insoluble and mixed enzymes*

	mg. ascorbic acid oxidized		
	I	II	III
Soluble enzyme	0·82	0·41	0·28
Insoluble enzyme	3·56	1·41	0·90
Mixed enzyme	4·32	1·80	1·15

It can be concluded that the activity of the mixed enzyme represents the total of the soluble and insoluble enzyme activities, and therefore the acetone-prepared enzyme can be employed in any comparative study of the enzyme contents of tea-leaf samples. Condensation is eliminated in the present method, and it is possible that the explanation of the discrepancy between these results and the previous ones is in some way connected with this condensation phenomenon.

Effect of concentration of enzyme. That the activity is proportional to the amount of the reacting enzyme is shown (Table 5) by an experiment in which the quantities of the enzyme were varied, keeping the other conditions the same. The period of reaction was, however, reduced to 30 min. in order to compare the activities while the reaction is proceeding briskly.

Table 5. *Effect of enzyme concentration*

Quantity of enzyme powder (mg.)	Activity (mg. ascorbic acid)
5	0·3
10	0·58
20	1·17
40	2·45
60	3·57

The ascorbic acid method can therefore be employed in tea-fermentation studies, especially in cases where it is required to assess or compare the enzyme activities. Such an application of the method has already been made in the study of enzyme activity in individual bushes.

Description of the proposed method and its advantages

Acetone-prepared enzyme powder (20 mg.) is weighed into a 50 ml. test-tube containing 5 ml. McIlvaine's buffer at pH 5·0 and 5 ml. catechol-

ascorbic acid substrate solution. The mixture is aerated for 1 hr., and 5 ml. filtrate are titrated in presence of 1 ml. 20% trichloroacetic acid with 2:6-dichlorophenol-indophenol solution standardized according to the method of Menaker & Guerrant [1939] for its ascorbic acid equivalent.

A control with the same quantities of enzyme and substrate but with 5 ml. 10% trichloroacetic acid solution instead of buffer is also run at the same time. The difference between the control value, which provides the initial amount of ascorbic acid, and the experimental result gives the amount of ascorbic acid oxidized during the reaction or, in other words, the enzyme activity. It has been established by a separate control that there are no autoxidation errors in the reaction at pH 5·0. No difficulty has been encountered in the dye titration of ascorbic acid due to the normal colour of either the enzyme extracts from tea leaf or its juice.

The iodometric method of measuring enzyme activity depends upon estimations of tannin by iodine titration, the limitations of which have been already stressed. The present method, on the other hand, has several advantages: (1) Both the chemicals used in substrate solution are of well-defined constitution and can be obtained in a high degree of purity. (2) The method is fairly rapid and does not involve the necessity of observing a standard time of reaction during titrations. (3) Greater accuracy is obtained not only in titrations but also from the fact that condensation errors are eliminated. (4) In iodometric titrations it was either necessary to obtain a completely tannin-free enzyme preparation or a control had to be run to determine the amount of buffer-extractable polyphenols. In the ascorbic acid method this is obviously unnecessary, as polyphenols are in fact added to the reaction mixture.

DISCUSSION

Condensation changes which follow the accumulation of *o*-quinones in a reaction mixture can take place between two or more molecules of *o*-quinones themselves or between one molecule of *o*-quinone and one or more molecules of unoxidized polyphenol. Such condensed products are oxidized by iodine relatively more slowly than the original polyphenols, and this fact is reflected in an apparent fall in their iodine titre. Some indication of this is afforded by our results on catechol oxidation by oxidase and peroxidase [Lamb & Sreerangachar, 1940*b*]. Oxidase activity produced very little change in iodine titre, whereas peroxidase produced a marked fall in titre, and it appeared that peroxidase activity resulted in the formation of more condensation products than does oxidase activity.

The criticism of Shaw's iodometric method for the volumetric estimation of tea tannin by Barua & Roberts [1940] is based on this question of condensation of tannin bodies after oxidation and the errors that may be introduced thereby. Reference to Table 3 shows that the conditions of optimum pH and temperature for the action of the tea-insoluble enzyme are almost the same when determined by the iodometric method previously used and by the present ascorbic acid method, which is independent of any interference due to condensation. Our previous conclusions, which were mostly based on the iodometric method, may not then be substantially affected by errors due to condensation.

An explanation of this agreement may perhaps be suggested. As condensation is a chemical reaction, it is reasonable to assume that the rate and amount of condensation of oxidized tannins will bear a definite relation to the amount of oxidized tannins present, which in its turn will be related to the activity of the enzyme. The fall in iodine titre due to the condensation factor would, therefore, vary in proportion to the enzyme activity itself, so that the method can still be employed in any comparative study of enzyme activities.

Further investigation is needed to amplify our present insufficient knowledge about this condensation process of oxidized tea tannin bodies. For example, it is not known whether or not the condensation phenomena are attended by any further oxygen uptake or even by simple dilatometric changes, in which case the manometric results on the fermentation of tea may be regarded as subject to an error.

SUMMARY

1. A new method of estimating oxidase activity in tea, by the use of ascorbic acid as substrate, has been described. The method is simple, accurate and avoids errors due to condensation of oxidized polyphenols, which was the chief drawback of the iodine titration method.

2. The values for the optimum pH and temperature of the insoluble enzyme are confirmed by this new method.

REFERENCES

Barua, D. N. & Roberts, E. A. H. [1940]. *Biochem. J.* **34**, 1524.
Ezell, B. D. & Gerhardt, F. [1940]. *J. agric. Res.* **60**, 89.
Lamb, J. & Sreerangachar, H. B. [1940*a*]. *Biochem. J.* **34**, 1472.
Lamb, J. & Sreerangachar, H. B. [1940*b*]. *Biochem. J.* **34**, 1485.
Menaker, M. H. & Guerrant, N. R. [1939]. *Industr. Engng Chem.* (Anal. ed.), **10**, 25.

Studies on the 'Fermentation' of Ceylon Tea

5. COMPARATIVE RATES OF OXIDATION OF DIFFERENT POLYPHENOLIC SUBSTRATES BY TEA-OXIDIZING ENZYMES

By H. B. SREERANGACHAR, *Tea Research Institute of Ceylon, Talawakelle*

(*Received* 8 *July* 1943)

The oxidizing-enzyme preparation employed in previous work was obtained from tea by grinding fresh leaf under acetone until the residue was almost completely free of all colouring constituents of the leaf.

Among the differences that exist between the *in vitro* action of this acetone-prepared tea enzyme and the *in vivo* reaction in the minced leaf are the rates of oxidation of different polyphenolic substrates. Thus, acetone-prepared enzyme oxidizes catechol about two or three times as rapidly as it does tea tannin [Lamb & Sreerangachar, 1940], whereas in the fermenting leaf-mince to which the polyphenolic substrates are introduced after completion of its initial fermentation, tea tannin is oxidized at about three times the rate of catechol oxidation [Harrison & Roberts, 1939].

There is obviously one main difference between the conditions that exist in the minced-leaf system prior to the addition of the polyphenolic substrates, and those obtaining in the acetone-prepared enzyme reaction system. In the former, the leaf polyphenols are, as a result of initial reactions, in various stages of oxidation and condensation, whereas in the latter such oxidized products of tea tannin are absent at the commencement of the reaction. The object of the present investigation is to find out the exact influence of these oxidized polyphenols on subsequent oxidation reactions, and to arrive at a clear distinction between the direct reactions of the enzyme and the possible indirect action of other substances present.

EXPERIMENTAL

Methods. The rates of oxidation of different polyphenols by tea enzymes, as shown by their oxygen uptake, were measured at 25° with Barcroft's apparatus [cf. Sreerangachar, 1941]. Observations included reactions in the fresh-leaf mince and also *in vitro* reactions of prepared enzyme and substrate. In the latter type of reaction, the enzyme powder was initially kept floating on the substrate solution in small 'pliofilm' boats which could be upset whenever desired by shaking the apparatus, thus bringing the enzyme and substrate into contact with each other. Any complication due to respiration changes was avoided by not introducing KOH into the central tube. Such a contingency arose only when fresh-leaf mince was used; for in the case of *in vitro* reaction mixtures the system was entirely free from living tissues. The polyphenolic substrate solutions employed were all of M/10 concentration. The amorphous tea tannin was prepared by the Tocklai modification of Shaw's method [1935], and all gallic acid was removed from it by extraction with dry ether.

Substrate specificity. Attention was first directed towards finding out the relative rates of oxidation

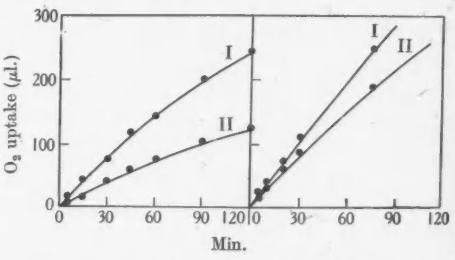

Fig. 1. Oxidation by acetone-prepared tea enzyme. I, catechol; II, tea tannin.

of catechol and tea tannin by the use of the same enzyme preparation. Acetone-prepared enzyme powder (50 mg.) was allowed to react on 2 ml. substrate solution to which 2 ml. McIlvaine's buffer solution at pH 5·0 were added. Fig. 1 represents the course of oxygen uptake by two such systems, and it will be seen that the rate of oxidation of catechol is higher than that of tea tannin, although the difference is not as great as that obtained previously with different enzyme preparations. This has been confirmed in several experiments.

The rate of oxidation of a few other substrates available here, namely, pyrogallol, ascorbic acid and quinol, were then studied in comparison with catechol and tea tannin (Fig. 2). The enzyme oxidizes catechol, tea tannin and pyrogallol, members of a class of substrates to which the enzyme is specific, more rapidly than it does ascorbic acid

and quinol, which do not belong to this specific class. The slight oxidizing action observed on ascorbic acid and quinol is due to the indirect influence of minute quantities of original polyphenols in the enzyme powder which resist complete extraction with acetone.

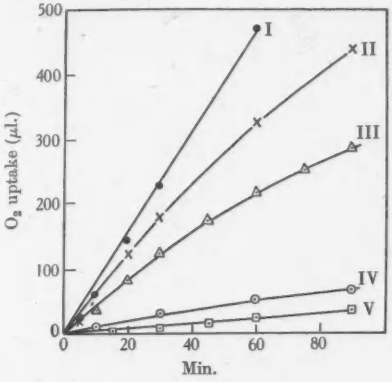

Fig. 2. Oxidation of polyphenols by acetone-prepared enzyme. I, catechol; II, tea tannin; III, pyrogallol; IV, ascorbic acid; V, quinol.

The oxygen uptake of the leaf-mince suspension came almost to a standstill after about 90–120 min. and the relative rates of oxidation of polyphenolic substrates were then measured by introducing them at this stage to the leaf-mince system. It was observed that tea tannin was oxidized more rapidly than catechol as shown in Fig. 3, I. Harrison & Roberts [1939] in Assam also found that tea tannin is oxidized about three times as rapidly as catechol with minced leaf.

Influence of oxidation products. A study of these results suggested that the tea polyphenols existing in different stages of oxidation and condensation as a result of the initial fermentation reaction in the leaf mince might probably be the chief cause of the difference between the results with acetone-prepared enzyme and those obtained with leaf mince. The interference of the oxidized products of tea polyphenols with the oxidation rates of polyphenols subsequently added might have been so pronounced as even to reverse the relative rates of oxidation of tea tannin and catechol. In order to test this hypothesis, fresh-leaf mince was repeatedly washed with water after its initial reaction, to remove the accumulated oxidation products, and the residue allowed to react on catechol and tea tannin. It was then found that the oxidation of tea tannin not only ceased to be faster than that of catechol but its rate was either the same or even lower than that of catechol as shown in Fig. 3, II.

The use of acetone, to remove the oxidation products from the leaf mince after the initial reaction, yields still better results, due to a greater extraction of these materials, though perhaps not so completely as in the preparation of enzyme from

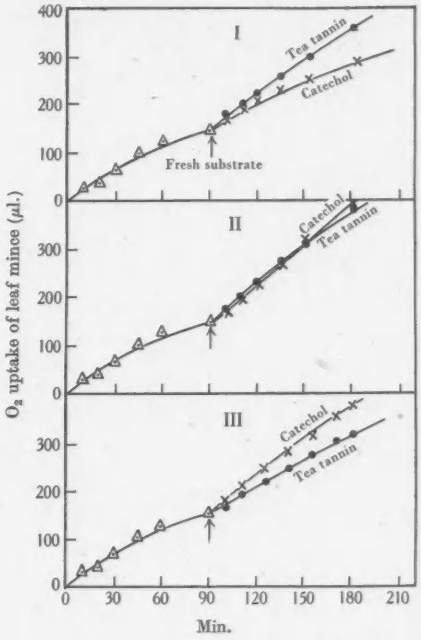

Fig. 3. O_2 uptake of leaf-mince suspensions with added substrates. I, reactions in presence of tannin oxidation products; II, ditto after removal of these by washing the tissues; III, ditto after removal by acetone extraction.

fresh leaf. Comparison with the results of water extraction reveals that acetone treatment does not appreciably inhibit the enzyme with respect to its rate of polyphenol oxidation. The rates of oxidation of catechol and tea tannin by the acetone-extracted residue, from leaf mince which had undergone the initial fermentation reaction, are in the same order as those observed in the case of acetone-prepared enzyme, although in the former case the difference between the oxidation rates of catechol and theotannin is not as great as in the latter. Fig. 3, III shows that catechol is being oxidized slightly more rapidly than tea tannin by the acetone-extracted residue of leaf mince which had previously been fermented.

Further confirmation of the influence of the tea-tannin oxidation products on subsequent oxidations of catechol and of tea tannin was obtained when these products, from another experiment, were

added to the reaction mixtures of the acetone-prepared enzyme on the one hand and catechol and tea tannin on the other. The relative rates of reaction at once changed, tea tannin being then oxidized more rapidly than catechol.

When this evidence concerning the influence of tea-tannin oxidation products on the subsequent oxidations of catechol and tea tannin had been gathered it was of interest to find out whether such behaviour was peculiar only to the tea-tannin oxidation products or whether it was generally true of the oxidized products of other polyphenols as well. The general scheme of the experiment was that the oxidized products of three phenolic bodies, catechol, quinol and tea tannin, should be added at the commencement of the reaction to systems each containing one of these polyphenols and the acetone-prepared enzyme. Comparisons of oxygen uptake results of two otherwise corresponding systems, with and without the addition of oxidation products, would reveal the effect, if any, of such additions. The oxidized products of catechol and tea tannin were obtained by oxidizing M/20 solutions of these polyphenols by acetone-prepared enzyme. It was not necessary to prepare experimentally the oxidized product of quinol, as pure *p*-quinone was available. A M/20 solution of *p*-quinone was used. Solutions (2 ml.) of these three oxidized polyphenols were added to each of the polyphenol-enzyme mixtures and the oxygen uptake measured by manometric methods. Fig. 4 represents the results obtained. The results shown in dotted lines (Fig. 4, A, D) were obtained with a separate sample of enzyme, as the sample used in the case of all other reactions became exhausted, a circumstance which cannot, however, affect the conclusions. These results are summarized in Table 1.

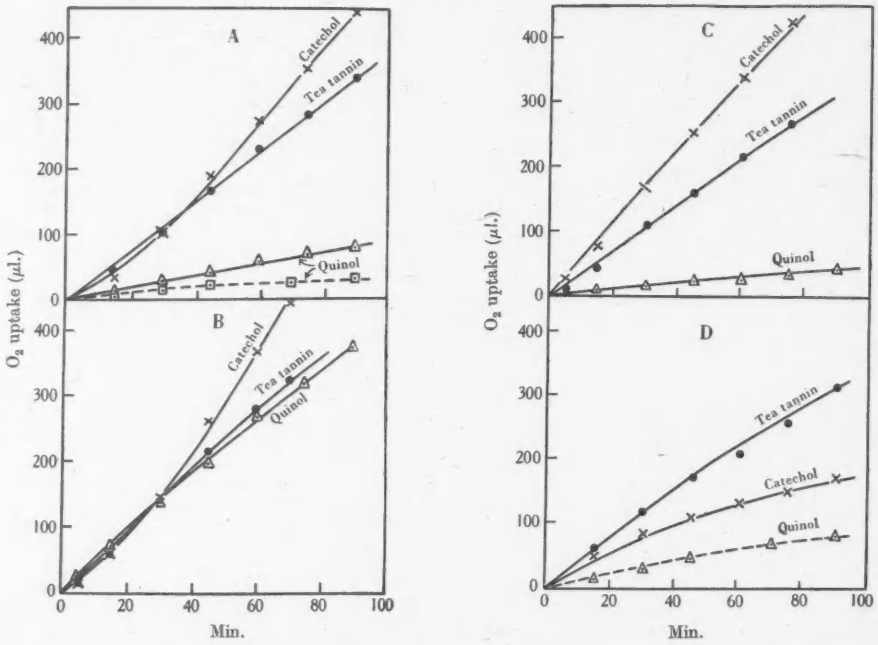

Fig. 4. A, oxidation of polyphenols by acetone-prepared enzyme from tea; B, reactions with added catechol oxidation products; C, ditto with added *p*-quinone; D, ditto with added tea-tannin oxidation products. Dotted lines in A and D represent reactions with a different enzyme.

Table 1. *Oxygen uptake during various polyphenol oxidations*

Product added	Polyphenol oxidation, μl. O_2/hr./mg. enzyme: Catechol	Tea tannin	Quinol	
Nil	5·5	4·6	1·2	0·4*
Catechol oxidation products	7·4	5·6	5·3	—
p-Quinone	6·8	4·4	0·6	—
Tea tannin oxidation products	2·6	4·3	—	1·2*

* A different sample of enzyme.

Catechol oxidation products accelerate oxidations of catechol and tea tannin almost to the same extent, while quinol oxidation is increased four to five times. *p*-Quinone accelerates catechol oxidation and does not much affect tea-tannin oxidation; but it markedly inhibits quinol oxidation. Tea-tannin oxidation products increase the rate of quinol oxidation nearly three times but inhibit catechol oxidation to less than half its original activity. Oxidation of tea tannin also suffers a slight inhibition. These results point to the general conclusion that the oxidation of polyphenols is markedly affected by the presence, at the beginning of the reaction, of certain polyphenol oxidation products. There appears to be, however, no correspondence between the polyphenol reaction affected and the polyphenol-oxidation products which influence the reaction. In particular, the reversal of the rates of oxidation of catechol and tea tannin in presence of oxidation products of the latter appears to be due to a marked depression of catechol oxidation, but not to an acceleration of the tea-tannin oxidation.

Corroboration of this point is noticeable in the results of fresh leaf-mince reactions and those with washed tissues. The oxygen uptakes during tea-tannin oxidation of 200 mg. leaf mince in 1 hr. (Fig. 3) are 135 and 150 μl. for unwashed and washed tissues respectively, while the corresponding figures for catechol oxidation are 98 and 163 μl. for the same unwashed and washed tissues. The marked inhibition of catechol oxidation by tea-tannin oxidation products is thus indicated.

When tea tannin is oxidized either in the fermenting leaf or *in vitro* by the prepared enzyme, the product consists of the oxidized tannins in a large range of condensations. In the present state of our knowledge about the chemistry of tea it is not possible to distinguish between the simple tea tannin-*o*-quinone and its various condensation products, or to ascertain their relative amounts in a solution of oxidized tea tannin. For this reason, the effect of tea-tannin oxidation products on subsequent polyphenol oxidations cannot be attributed singly either to the one or the other of these constituents. The following experiment, however, affords some evidence on this point. Condensation in a solution of oxidized tannin can be increased by rendering the solution alkaline, and it was desired to find out whether such an increase in the condensation products would materially affect the results. Five ml. of an oxidized tea tannin solution (M/20) were made alkaline to pH 9·5–10·0, boiled and cooled, and the pH readjusted to 5·0. This solution and 5 ml. of original solution were both made up to 10 ml., and 2 ml. of each were added to the oxidation-reaction systems of catechol and tea tannin.

It can be concluded from the results (Fig. 5) that increasing the condensation products has no significant effect on the rate of tannin oxidation, while it slightly enhances the inhibition of catechol oxidation. Therefore, the inhibition effect observed in catechol oxidation by addition of tannin-oxidation products is probably to be associated more with the

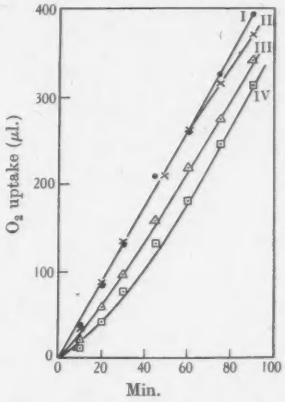

Fig. 5. Oxidations of catechol and tea tannin in presence of tea-tannin oxidation products. I, tea-tannin oxidation, lower condensed products; II, ditto, higher condensed products; III, catechol oxidation, lower condensed products; IV, ditto, higher condensed products.

higher condensation products of tannin-*o*-quinone than with the simpler tannin-*o*-quinone itself. The difference between the rates of catechol oxidation with the two concentrations of condensed products is, however, small, but it may probably be due to the fact that the original solution of oxidized tea tannin which was standing for 24 hr. already contained an effective amount of condensed tannin-oxidation products, which might have been increased only slightly on the solution being rendered alkaline.

Reaction in a mixture of substrates. When a single enzyme oxidizes two substrates, the rate of oxidation of their mixture generally falls between the two individual values owing to the competition of substrates for the enzyme. An investigation of the action of the acetone-prepared enzyme on a mixed substrate of catechol and tea tannin showed (Fig. 6) that the initial rate of oxidation of the mixture was intermediate between the rates of individual oxidations of catechol and tea tannin. This is in accordance with the above general rule and indicates that a single enzyme is concerned in the reaction. Towards the end of the reaction, however, the accumulation of the oxidation products begins to exert its influence and tends to depress the rate of reaction.

Addition of catechol to the fermenting leaf leads to conditions in which the same tea enzyme is acting simultaneously both on catechol and tea tannin. The rate of oxidation would then be intermediate between the oxidation rates of the two

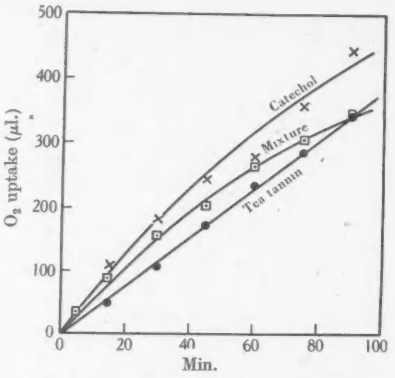

Fig. 6. Oxidation of catechol, tea tannin and their mixture by acetone-prepared enzyme.

individual substrates. Harrison & Roberts [1939], however, found in a similar experiment that the rate of oxidation of the mixture was higher than that of the individual substrates. In arriving at this conclusion they made use of their previous result that catechol is oxidized at about one-third the rate of tea tannin, a result which was obtained without taking into consideration the presence and, therefore, the influence, of tannin-oxidation products. But, in view of the facts shown in the preceding section, the validity of this conclusion is open to doubt. When the individual rates of oxidation of catechol and tea tannin are investigated under conditions free from any extraneous interference, it is possible that the rate of oxidation of the mixture of substrates by the fermenting leaf system is actually intermediate between the two individual values.

DISCUSSION

Roberts [1941] has suggested that the difference in the rates of oxidation of catechol and tea tannin observed at Tocklai and St Coombs is due to the presence of two different enzymes in the materials derived from the two different sources. In the literature there is, no doubt, some evidence for the presence of more than one oxidase system in the same plant tissue [Boswell & Whiting, 1938; Marsh & Goddard, 1939]. But in this instance such a suggestion seems unnecessary, since this particular disagreement between St Coombs and Tocklai is due more to a difference in technique than to the nature of the materials. The reaction in the minced leaf as employed by Roberts is complicated by the accumulated tannin-oxidation products, which exert some influence on the course of the subsequent reactions.

SUMMARY

1. The relative rates of oxidation of catechol, tea tannin, pyrogallol, quinol and ascorbic acid by the tea enzyme prepared with acetone have been studied by manometric methods.

2. The discrepancy in the comparative rates of oxidation of catechol and tea tannin between our results and those of Assam workers has been shown to be due to an inhibitory effect, under the conditions of Assam experiments, of the condensation products of oxidized tea tannin on the oxidation of catechol. In view of this, the supposition of two different enzymes in Assam and Ceylon seems unnecessary.

3. It is shown that the presence of oxidized products of polyphenols at the commencement of a subsequent polyphenol oxidation reaction affects, in general, the course of such reaction.

4. The action of the enzyme prepared with acetone in a mixture of catechol and tea-tannin substrates has yielded results consistent with the view that both these substrates are oxidized by a common enzyme, namely, the tea polyphenol oxidase.

The author wishes to thank Dr R. V. Norris, Director, Tea Research Institute of Ceylon, for permission to publish these results, Mr J. Lamb, Biochemist, for his kind interest and advice in this investigation, and Dr T. Eden, Agricultural Chemist, for criticizing the manuscript.

REFERENCES

Boswell, J. G. & Whiting, G. C. [1938]. *Ann. Bot., Lond.*, N.S., **2**, 847.

Harrison, C. J. & Roberts, E. A. H. [1939]. *Biochem. J.* **33**, 1408.

Lamb, J. & Sreerangachar, H. B. [1940]. *Biochem. J.* **34**, 1472.

Marsh, P. B. & Goddard, D. R. [1939]. *Amer. J. Bot.* **26**, 724.

Roberts, E. A. H. [1941]. *Biochem. J.* **35**, 1209.

Shaw, W. S. [1935]. *Bull. Uni. Plant. Ass. South India*, **4***a*.

Sreerangachar, H. B. [1941]. *Biochem. J.* **35**, 1106.

Studies on the 'Fermentation' of Ceylon Tea

6. THE NATURE OF THE TEA-OXIDASE SYSTEM

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Recent investigations on tea fermentation, both in Ceylon and Assam, have led to distinct differences of opinion regarding the nature of the oxidase system that is responsible for fermentation. After modifying his earlier views on this question at least twice, Roberts [1939; 1940] has now identified the tea-oxidizing enzyme with cytochrome oxidase. In previous studies from these laboratories [Lamb & Sreerangachar, 1940], not only have objections to the cytochrome theory of tea fermentation been presented, but also definite evidence for the view that tea oxidase is a polyphenol oxidase with an established specificity for *o*-dihydric phenols.

Despite this, the considerable body of indirect evidence that Roberts adduced in favour of his theory made it appear for the moment a reasonable assumption, but further work has shown that alternative explanations could be found for most of his material observations. Perhaps the most serious objection to the cytochrome theory of tea fermentation is that so far there is no direct evidence for the presence in tea leaf of cytochrome and cytochrome oxidase, which seems quite essential for the acceptance of the theory. The present investigation was, therefore, undertaken with a view to obtaining some definite information which would serve either to establish or confute the presence of these components in tea.

Spectroscopically, cytochrome is easily detected in its reduced state by its characteristic absorption spectrum. Since chlorophyll may interfere in the observations on the leaf it may be extracted by solvents such as acetone. Any possible inactivation of the oxidase in the process does not affect either the content or the character of cytochrome. Following this procedure, Yakushiji [1935] has reported seeing the α-bands of cytochrome *b* and *c* in acetone-extracted spinach leaves. Choosing the basal portion of the stem, probably with a view to obviating the chlorophyll interference, Roberts [1941], with Miss K. Bhagvat, claims to have demonstrated the presence of cytochrome in the tea plant. They appear to have observed an absorption band corresponding to the '*b*' band of cytochrome in the green region of the spectrum. It is not clear whether it is the component *b* or the spectral band β that they have observed, and it is noteworthy that other bands of either cytochrome *b* or *c* (it cannot be component α as its β band is unknown) are not observed.

Spectroscopy can also be used in detecting the presence of cytochrome oxidase. On oxidation by the enzyme the sharp absorption bands of reduced cytochrome give place to the diffuse bands of oxidized cytochrome which are hardly perceptible in the micro-spectrophotometer. Disappearance of reduced cytochrome bands would therefore indicate the action of the cytochrome oxidase.

Before proceeding to describe the experimental evidence in the present investigation, a brief reference may be made to those points of dissension between workers here and in Assam not previously discussed by us.

Methods of investigation. In earlier work we have relied mostly on the titrimetric methods in the estimation of the tea-oxidase activity, and we have recognized the limitations of the method. Roberts [1941] has criticized this method on the ground that the iodine titre is affected by condensation of *o*-quinones produced, but has admitted that these errors are likely to be very small.

However, an alternative method of estimating enzyme activity, using ascorbic acid as substrate as described in Part 4 of this series, has yielded identical results as regards the optimum pH and temperature of the enzyme reaction. Therefore condensation errors in the iodine titration method could only be very small, and in a comparative experiment the conclusions are practically unaffected by these errors. It is interesting to note in this connexion that Roberts [1941] has himself obtained results of optimum pH (5·4) and temperature (29°) which are very close to those found by us. Such correspondence detracts very considerably from the relevance of his criticisms of the accuracy of our method. From the point of view of our present contention about the specific nature of the oxidase, the quantitative accuracy of the method is relatively less important. It is sufficient that the direct oxidase activity of the enzyme on polyphenols has been demonstrated beyond doubt.

The study of simpler component reactions affords an easy approach to the understanding of the most complicated biological processes *in vivo*. Preparation of purified enzyme and a study of its reaction *in vitro* on pure chemical substances is therefore a fruitful line of investigation [e.g. Hussein & Cruess, 1940], and we have always followed this course in our study of the mechanism of tea fermentation and the substrate specificity of the enzyme.

Substrate specificity. The importance of this property of the enzyme cannot be over-emphasized. Roberts's summing up [1941] of evidence on this point unfortunately lacks clarity. We admit that substrates like *p*-phenylene-

diamine and ascorbic acid, when added to fermented tea-leaf suspensions, are doubtless oxidized. But is it due to the existence of a cytochrome system? Is it not possible that the very reactive *o*-quinone reacts with these added substrates while it is itself being reduced to polyphenol? The enzymic nature of stimulation of O_2 uptake would thus be maintained and tea polyphenol would act as a carrier in the reaction. Thus the oxidations of ascorbic acid and *p*-phenylene-diamine would be only secondary reactions, and the fact that polyphenol-free enzyme preparations fail to oxidize directly these substances as well as quinol lends further support to this view.

Our enzyme preparations display a clear-cut specificity for *o*-dihydric phenols, and such a marked specificity is incompatible with the presence of cytochrome + cytochrome oxidase. However crude our enzyme preparation may be, it must be regarded as relatively pure when compared with tea-leaf mince and, unlike the latter, its reaction on substrates is free from the interference of tea polyphenols. To that extent, our results on substrate specificity of the oxidase system are more reliable than those of Assam workers.

Effect of organic solvents. Acetone inhibition seems to be characteristic of cytochrome oxidase, and so Roberts has inferred that our acetone-prepared enzyme would have undergone a substantial loss in activity. Nevertheless, we have obtained strong activity of this acetone-prepared enzyme on catechol and other *o*-dihydric phenols. The assumption that tea leaf originally contained cytochrome oxidase and that it was considerably inactivated during acetone treatment would be consistent with the failure of the enzyme to oxidize quinol and *p*-phenylene-diamine. But how are we to explain the strong activity on catechol unless, of course, it is granted that the leaf originally contained polyphenol oxidase also?

It is likely that besides cytochrome oxidase other plant oxidases are also inhibited to a different degree by acetone. For instance, Srinivasan [1936] has shown that ascorbic acid oxidase is inhibited by acetone. Although we have found the greater part of the original enzyme activity in our preparations, it is possible that they may not have retained the same polyphenol-oxidizing activity as in fresh leaf.

Oxygen tension. Further inuirect evidence for the cytochrome theory consists in Roberts's observation that the initial rate of O_2 uptake by fermenting tea leaf is markedly accelerated by an increase in the O_2 tension. But it is doubtful whether this is an established property of cytochrome oxidase. Green [1940] states that 'the rate of oxidation of the ferrocytochrome by molecular oxygen in presence of the enzyme is practically independent of the partial pressure of O_2'. In fact, since the respiration of yeast and other micro-organisms is found to be independent of O_2 tension, this is taken as evidence for the functioning of cytochrome oxidase in aerobic systems.

Evidence for the cytochrome theory has also been inadequate on other points. For instance, the failure to depress the rate of O_2 uptake when catechol is added to fermenting tea leaf cannot be considered as proof for the carrier nature of the tea oxidase, as is shown in Part 5 of this series. The existence of both soluble and insoluble enzymes in tea makes any evidence based on solubility inconclusive. If the enzyme is considered too insoluble to be polyphenol oxidase, it is also too soluble to be cytochrome oxidase.

EXPERIMENTAL

Spectroscopic investigations

Presence of cytochrome in tea. Employing a micro-spectrophotometer we made observations on the finely ground tissues in presence of both sodium succinate and $Na_2S_2O_4$. Contrary to Roberts's results, examination of the basal portion of the stem in tea plant did not give any positive indications for the presence of cytochrome. Similarly, other tissues of the plant, when separately collected and tested, failed to display any characteristic absorption bands which could be identified with those of cytochrome.

No cytochrome could be detected in the acetone-extracted tea leaf from which the possible interfering substances like chlorophyll and tannin were removed by solvent extraction. Attempts to prepare cytochrome *c* from leaf by the method of Keilin & Hartree [1937] yielded negative results. Both Ceylon and Indian tea leaves were used in this investigation.

From certain considerations it seemed reasonable to expect the presence of cytochrome *c* in our enzyme preparations. First, Yakushiji's [1935] findings made the presence of cytochrome probable in the acetone-extracted tea leaf, from which our oxidase preparations were obtained. Secondly, the ammonium sulphate saturation employed in the preparation of cytochrome *c* from ox heart [Theorell, 1936] was also the method used for the purification of the soluble enzyme, as will be described later. Furthermore, since Roberts attributed the oxidizing activity of the enzyme preparations to the cytochrome system, it was reasonable to expect the presence of cytochrome *c* in highly active enzyme concentrates. But it was found that none of these preparations, when spectroscopically examined, displayed any specific absorption bands. Figs. 1 and 2 give the absorption spectra of a highly active tea oxidase solution. The results in the visible region of light (Fig. 2) were obtained in a Gaertner's polarizing spectrophotometer, while for a study of absorption in the ultra-violet region (Fig. 1) a Hilger's instrument was employed. 10 mm. cells were used in both cases.

It will be seen that there is no selective absorption either in the visible region or in the ultra-violet region studied, and therefore the preparation does not contain cytochrome. The component cytochrome a_3, which Keilin & Hartree [1939] identify with cytochrome oxidase, has a characteristic absorption (430 and 590mμ), and most of the other haematins have a strong absorption in the extreme violet between 400 and 420mμ [Dixon, Hill & Keilin, 1931]. But the spectrum of the tea-oxidase solution cannot be related to any of these.

Detection of cytochrome oxidase. The detection of cytochrome oxidase was based on its *in vitro* reaction on cytochrome *c*, which could be followed in the spectrophotometer. In view of the possible complications that would be introduced, the fresh-leaf mince or the finely ground tissue could not be used for this investigation. On the other hand, the acetone-prepared enzyme was considered a suitable material; for, if the observed activity of the preparation was attributed to the cytochrome system, it should be possible to demonstrate cytochrome oxidase in it.

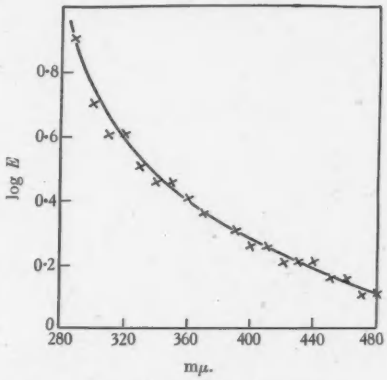

Fig. 1. Ultra-violet absorption spectrum of tea oxidase.

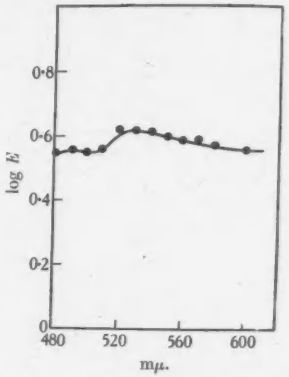

Fig. 2. Visible region absorption spectrum of tea oxidase.

Preparation of tea oxidase. In order to minimize the inactivating effect of the solvent on the enzyme, the acetone extraction of the oxidase was carried out at about $-20°$ in a bath of ice and salt under the following conditions, which are a refinement of those previously used by us. The leaf material was derived from young tea plants in the garden of the Indian Institute of Science, Bangalore. About 10 g. of fresh leaf were finely ground up, soon after plucking, with 30 g. of ice crystals and some iron-free sand and then introduced into a beaker which was kept immersed in a bath of ice and salt at $-20°$. Acetone (300 ml.), previously cooled to the same low temperature, was then added with vigorous stirring. The acetone extract was sucked off by means of an inverted thistle funnel, packed at the mouth with glass-wool, and immersed in the beaker. Renewal of cold acetone and repetition of the extraction three or four times rendered the preparation almost completely free of polyphenol, as tested by $FeCl_3$ and also by self-digestion of the preparation in the Warburg manometer. The material was quickly dried *in vacuo* and then extracted with 100 ml. phosphate buffer at pH 7·0, under cold conditions. After filtration the extract, which was highly active towards catechol, was brought to pH 5·0 with acetic acid and mixed with sufficient ammonium sulphate to saturate to the extent of 1/3. From the time of plucking up to this stage, when the enzyme may be expected to be more stable, the time taken was only 4–5 hr., and since the operations were, as far as possible, done in the cold, the inactivation of the enzyme must have been minimal during the preparation. The fluid was left overnight in the ice-box.

The slight precipitate that appeared was then filtered off, and to the filtrate more ammonium sulphate was added to bring it to half-saturation. There was a further precipitate which was also filtered off, and the filtrate was then saturated with ammonium sulphate, when again there was a precipitate. The three precipitates obtained were dispersed in water and dialyzed in the cold against running distilled water. The dialyzed suspensions, obtained by one-third or one-half saturation, contained for the most part inert matter with comparatively little enzyme, and they were therefore discarded. The precipitate at full saturation gave, on dialysis, a pale yellow solution constituting a very active preparation of oxidase. This could be preserved in the refrigerator for a considerable period without appreciable loss of activity.

The residue, after extraction of soluble enzyme from the acetone-prepared leaf powder, was further ground into a fine suspension and also preserved in the ice-chest.

Experiments with reduced cytochrome c and cytochrome oxidase from heart muscle. Cytochrome *c* was prepared by the method of Keilin & Hartree [1937], and it was found that goat's heart proved a better source than ox heart. The dialyzed cytochrome solution was preserved in the ice-chest. Reduction

of cytochrome was carried out by passing H_2 in presence of either freshly prepared palladinized charcoal [Hartung, 1928] or of platinized asbestos (E. de Haen's preparation). An active preparation of cytochrome oxidase was also obtained from fresh goat heart muscle by the method of Keilin & Hartree [1938].

The heart-muscle oxidase readily catalyzed the oxidation of reduced cytochrome, with consequent disappearance of the latter's characteristic absorption bands in the microspectrophotometer. But the tea-oxidase solution prepared as above, when added to the reduced cytochrome substrate, did not have any effect whatever on the absorption bands, which remained practically steady for about 24 hr. It must, therefore, be concluded that the specific cytochrome oxidase was absent from the tea-oxidase preparation. The activity towards catechol, on the other hand, was very marked, as determined by its rapid oxygen uptake ($-Q_{O_2} = 170$).

A suspension of the insoluble enzyme also gave similar spectroscopic results, which points to the same conclusion as in the case of the soluble enzyme.

The course of reaction of these enzyme preparations on reduced cytochrome, as compared with that of the heart oxidase, was further followed in the polarizing spectrophotometer. 0·5 ml. enzyme was allowed to react with 2·5 ml. reduced cytochrome solution (3×10^{-4} M) for 2 hr. at room temperature, and spectroscopic determinations were carried out in 10 mm. cells.

It will be seen from Figs. 4 and 5 that the absorption characteristics of reduced cytochrome remained unaffected in presence of tea-oxidase preparations, while heart oxidase (Fig. 3) brought about a complete change. In the case of heart enzyme and tea-insoluble enzyme the reaction mixtures had to be filtered before taking measurements in the spectrophotometer, and the slight shift in the curve in the latter instance is perhaps due to the absorption of cytochrome *c* on the solids of the tea-residue suspension.

The degree of oxidation of reduced cytochrome *c* is related to the fall in the intensity of its absorption bands. The kinetics of the oxidation can therefore be followed by measuring in the above reactions the extinction coefficients at 550 mμ, the maximum of reduced cytochrome *c*. Fig. 6 represents these data, and it will be seen that whereas the relative concentrations of reduced and oxidized cytochrome remain unaltered in the case of tea oxidase, the heart oxidase action results in a rapid fall in the reduced cytochrome concentration.

These results show that, judged on its specificity of reaction, tea oxidase cannot be identified with cytochrome oxidase.

Roberts [1941] has reported observing the reduced cytochrome bands when cytochrome *c* and succinate were added to the finely ground tea tissue and that these bands disappeared on vigorous shaking. In this reaction there was no need for any extraneous addition of cytochrome *c*, since he had

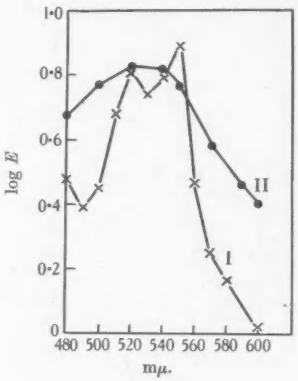

Fig. 3.

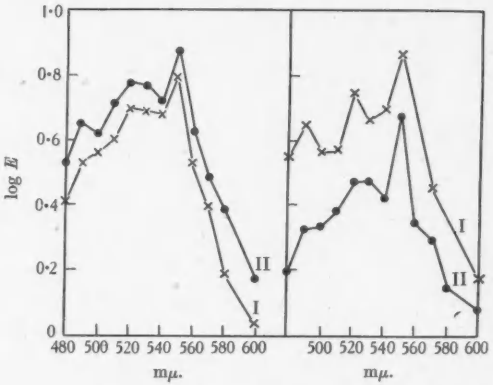

Fig. 4. Fig. 5.

Fig. 3. Oxidation of cytochrome by heart oxidase. I, absorption spectrum of reduced cytochrome; II, reduced cytochrome *c* + heart-muscle enzyme.

Fig. 4. Action of tea-soluble enzyme on reduced cytochrome. I, reduced cytochrome; II, reduced cytochrome + tea-soluble enzyme.

Fig. 5. Action of tea-insoluble enzyme on reduced cytochrome. I, reduced cytochrome; II, reduced cytochrome + tea-insoluble enzyme.

already observed cytochrome in the tissue by its '*b*' band. Further, he has remarked elsewhere in his paper [Roberts, 1941] that when cytochrome *c* of ox heart is added to tea-tissue suspension it would combine with tea tannin and would thus be 'inactivated', thereby meaning perhaps an irreversible chemical change in cytochrome. If that is so, the disappearance of the reduced cytochrome bands may not be due to the presence of cytochrome oxidase. In our experiments we have found that

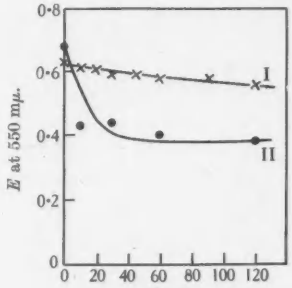

Fig. 6. Oxidation of cytochrome. I, tea oxidase; II, heart oxidase.

when reduced cytochrome is added to tea-tissue suspensions there is a rapid colour change from original red to a dirty yellow. The spectrum of the filtered liquid does not correspond to that of oxidized cytochrome and it cannot be reduced again either by Pd and H_2 or by $Na_2S_2O_4$. The change suffered by reduced cytochrome is not, therefore, a simple reversible oxidation, and under the circumstances the presence of cytochrome oxidase in the tissue becomes doubtful.

Manometric investigations

The oxygen uptake measurements were made in Warburg manometers with all necessary controls. All reactions were carried out at pH 7·0, total volume 2·5 ml., and temperature 30°. The first point established was the marked activity of the enzyme solution on catechol and its inactivity on reduced cytochrome *c* as substrate (Table 1).

Reduced cytochrome had not been reacted upon, as was shown by its absorption bands when tested at the end of the reaction.

In the *in vitro* reconstruction of the well-known respiratory system of cytochrome *c*, cytochrome oxidase, succinate and its dehydrogenase, it was desired to find out if the enzymes in the system could be replaced by tea-oxidase preparation. Results of oxygen uptake by the substrate, reduced

Table 1. *Oxygen uptake of tea oxidase with catechol and cytochrome*

Oxygen uptake when 0·5 ml. of diluted enzyme (original solution ×4) was allowed to react on 1 ml. of catechol solution (M/20) and 1 ml. of reduced cytochrome solution, respectively, in presence of 1·0 ml. of phosphate buffer at pH 7·0.

Time period (min.)	5	10	20	30	45	75	90	120	150
Catechol substrate (μl. O_2)	5	12	22	35	54	91	112	145	184
Reduced cytochrome substrate (μl. O_2)	3	1	4	1	0	0	0	0	4

cytochrome + succinate, in presence of tea-soluble and insoluble enzymes on the one hand and the heart enzyme on the other are given in Table 2.

Table 2. *Oxygen uptake of different enzyme systems on cytochrome c + succinate*

In the case of the heart enzyme the cytochrome concentration was $1·2 \times 10^{-5}$ M; in the case of tea enzymes $6·0 \times 10^{-5}$ M. 0·5 ml. enzyme and 0·5 ml. 1% succinate were used in all cases.

Time period (min.)	5	20	60	90	120	150
Tea-soluble enzyme (μl. O_2)	0	3	3	3	6	—
Tea-insoluble enzyme (μl. O_2)	0	4	8	6	3	—
Heart enzyme (μl. O_2)	4	—	46	59	90	110

Failure of tea enzyme to stimulate oxygen uptake in the above reactions may be due to the absence of either cytochrome oxidase or succinic dehydrogenase or both in the tea-oxidase preparations. If this mixture of reduced cytochrome *c* + succinate + tea oxidase is supplemented by a further factor, namely, succinic dehydrogenase free from cytochrome oxidase, oxygen uptake will then proceed, provided that tea oxidase contains cytochrome oxidase. But succinic dehydrogenase and cytochrome oxidase are invariably concomitant in animal tissues, and attempts to separate them by ultracentrifugation, heat inactivation and so on were not successful. However, if tea oxidase contains cytochrome oxidase, its addition to a succinic dehydrogenase preparation with only a weak cytochrome oxidase activity should result in an acceleration of the rate of oxygen uptake of the substrate mixture because of an increase in the cytochrome oxidase concentration. A succinic dehydrogenase preparation obtained from goat heart muscle by the method of Ogston & Green [1935] was found to have only a poor cytochrome oxidase activity, but strong succinic dehydrogenase activity, as tested by the usual methylene-blue technique. Addition of this preparation to the system 'reduced-cytochrome + succinate + tea oxidase' did not increase the rate of O_2 uptake in the reaction to any appre-

ciable extent. For the sake of clarity the comparative oxygen uptake values are summarized in Table 3.

Table 3. *Oxygen uptake by various reaction systems*

Reaction	Oxygen uptake μl./hr.
Catechol + tea oxidase	198
Reduced cytochrome *c* + tea oxidase	—
,, + succinate + tea oxidase	3
,, + ,, + succinic dehydrogenase preparation	9
,, + ,, + ,, ,, + tea oxidase	11

The absence of stimulation of oxygen uptake by the tea enzyme in all reactions in which cytochrome *c* formed the substrate must be interpreted as due to lack of cytochrome oxidase. The results obtained demonstrate unequivocally that tea oxidase cannot be identified with cytochrome oxidase. But, on the other hand, since catechol is readily oxidized by the enzyme, there can be little doubt about its polyphenol oxidase nature.

DISCUSSION

A similarity in a few properties led Roberts to propose his cytochrome theory of tea fermentation. Since then, these similarities have been shown to be spurious, and according to his own findings there are serious and 'very marked differences' between tea oxidase and animal-cytochrome oxidase. But these differences he attributes to a dissimilarity that may exist between plant-cytochrome oxidase and animal-cytochrome oxidase. Unfortunately, very little is known to date about plant-cytochrome systems, and until they are fully investigated it may be premature to base any conclusions on them.

In explaining the failure of ox-cytochrome *c* to accelerate tea-tannin oxidation, Roberts has gone even further. He has assumed tea-leaf cytochrome to be different from the ox-heart cytochrome in that the latter is inactivated by combination with tea tannin while the former is endowed with some type of immunity from tannin. If this be true it seems to be something specific to tea and perhaps to other tanniferous plants; for Brown & Goddard [1941] have observed that ox-cytochrome *c* does stimulate the oxidation of *p*-phenylene-diamine by the cytochrome oxidase present in wheat embryos. However, Roberts's postulate, of the presence in tea of a cytochrome immune to tea tannin, is reminiscent of his previous postulate of an immune peroxidase and protective substances, which has not as yet been confirmed.

Certain differences pointed out in the oxidase systems concerned in tea fermentation in Ceylon and Assam appear, on closer examination, to be a consequence of the use of different techniques in the two places. For instance, in our previous studies we have shown that catechol is oxidized by our enzyme preparations at a higher rate than tea tannin, and this has been confirmed. The results of Assam workers with their material appear to be exactly the reverse. The difference is only apparent, not real, because the conditions were not the same in both cases; the Assam experiments were carried out with minced leaf in which tea-tannin oxidation products had accumulated, but these were eliminated in our reaction mixtures. The tannin-oxidation products do interfere with the rate of subsequent oxidations by the enzyme, as is fully discussed in Part 5 of this series. Such an interference is in fact found to be responsible for the reversal in the rates of oxidation of catechol and tea tannin as observed by Assam workers. The suggestion of a difference in the nature of Assam and Ceylon enzymes receives, therefore, no support from the above consideration. A point of some interest in this connexion is that the present investigation also included the enzyme derived from tea plants raised on Indian seeds and grown under Indian conditions.

The spectroscopic and manometric evidence obtained in the investigation goes to show conclusively that tea oxidase cannot be identified with cytochrome oxidase, which forms the keystone of the entire structure of the cytochrome theory of tea fermentation. Absence of cytochrome further justifies the rejection of the theory. On the other hand, our original finding that the tea-fermentation enzyme was a polyphenol oxidase needs no modification whatever. The mechanism of tea fermentation based on this finding, whilst not so complex as the cytochrome theory, is consistent with the main facts so far observed.

SUMMARY

1. Attempts to demonstrate directly by spectroscopic methods the presence of cytochrome in tea have been unsuccessful.

2. Spectroscopic investigation of highly active tea-oxidase preparations showed that the enzyme could not be identified with cytochrome oxidase. Manometric results have confirmed this conclusion.

3. Some of Roberts's previous results have been reviewed in the light of the later work and certain

differences have been satisfactorily accounted for. From the evidence presented, Roberts's cytochrome theory of tea fermentation cannot be considered valid.

This investigation was carried out at the Indian Institute of Science, Bangalore, where spectroscope facilities were available. The author is indebted to Sir J. C. Ghosh, Director, Indian Institute of Science, Bangalore, for laboratory accommodation and to Mr M. Srinivasaya, Head of the Fermentation Technology Section at the Institute, for much helpful advice and discussion. Thanks are also due to Dr R. V. Norris, Director, Tea Research Institute of Ceylon, for permission to publish these results and to Mr J. Lamb, Biochemist, for his kind interest in the investigation.

REFERENCES

Brown, A. H. & Goddard, D. R. [1941]. *Amer. J. Bot.* **28**, 319.
Dixon, M., Hill, R. & Keilin, D. [1931]. *Proc. roy. Soc.* B, **109**, 29.
Green, D. E. [1940]. *Mechanism of Biological Oxidations*, p. 31. Cambridge: University Press.
Hartung, W. H. [1928]. *J. Amer. Chem. Soc.* **50**, 3372.
Hussein, A. A. & Cruess, W. V. [1940]. *Food Research*, **5**, 65.
Keilin, D. & Hartree, E. F. [1937]. *Proc. roy. Soc.* B, **122**, 298.
Keilin, D. & Hartree, E. F. [1938]. *Proc. roy. Soc.* B, **125**, 171.
—— —— [1939]. *Proc. roy. Soc.* B, **127**, 167.
Lamb, J. & Sreerangachar, H. B. [1940]. *Biochem. J.* **34**, 1472.
Ogston, F. J. & Green, D. E. [1935]. *Biochem. J.* **29**, 1983.
Roberts, E. A. H. [1939]. *Biochem. J.* **33**, 842.
—— [1940]. *Biochem. J.* **34**, 500.
—— [1941]. *Biochem. J.* **35**, 1209.
Srinivasan, M. [1936]. *Biochem. J.* **30**, 2077.
Theorell, H. [1936]. *Biochem. Z.* **285**, 207.
Yakushiji, E. [1935]. *Acta Phytochim., Tokyo*, **8**, 325.

Studies on the 'Fermentation' of Ceylon Tea

7. THE PROSTHETIC GROUP OF TEA OXIDASE

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Earlier investigations in this laboratory on the specific nature of the oxidizing enzymes in tea [Lamb & Sreerangachar, 1940; Sreerangachar, 1943] have shown that the enzyme belongs to the family of polyphenol oxidases of which the potato oxidase and the mushroom oxidase are well-known members. The work of Kubowitz [1937; 1938] and of Keilin & Mann [1938] respectively on these enzymes has established that these polyphenol oxidases are Cu-protein compounds, and undergo a cycle of transformation from cupric to cupro form and vice versa, in the course of their catalytic activity. Cu is also found in the prosthetic groups of other oxidative enzymes such as laccase, ascorbic acid oxidase and tyrosinase, but the specific protein carrier in each of these enzymes is considered to be different. The Cu-proteins, unlike the Fe-porphyrin protein enzymes, show no specific absorption bands in the visible region of the spectrum, the absorption, if any, being only diffuse. A spectroscopic examination of tea oxidase has failed to reveal any characteristic absorption bands [Sreerangachar, 1943], and the enzyme is, therefore, analogous in this behaviour to Cu-proteins.

Consequent on the finding that tea oxidase was a polyphenol oxidase with respect to its substrate specificity, further investigations of the material and chemical nature of this enzyme became desirable, and it was thought that a study of the prosthetic group was a fruitful line to follow in the first instance. In particular, it was intended to see whether, like the other known examples of polyphenol oxidases, tea-polyphenol oxidase was also a Cu-protein compound.

Cu is easily detected in tea-oxidase preparations, but it was found that the crude preparations also contained Mn and Fe. As these two metals are also frequently associated with certain oxidizing enzymes, the possibility of their forming the active group of tea oxidase had to be considered in the early stages. According to Bertrand & Mallèvre [1895], there exists a close connexion between Mn and laccase, and Shaw & Jones [1935] have emphasized the importance of Mn in the study of the oxidative enzyme in tea. That the fresh tea leaf contains appreciable amounts of Mn can be seen by the fact that the ash is generally green because of the presence of manganous salts, which readily change over to the characteristic permanganate colour when wetted with oxidizing solutions. But in spite of this, the acetone-prepared enzyme contains only a fraction of the original amount (200–400 μg./g.). Further, Mn is almost completely absent even in partially purified preparations of enzyme and there-

fore it clearly cannot be associated with the active group of the enzyme.

The importance of Fe lies in the fact that, should it be possible to establish its constant association with tea oxidase, the latter would have to be classified along with Fe-containing enzymes or haemochromogens. This would not, however, be quite consistent with the chemical constitution of a polyphenol oxidase as understood at present. Tea-enzyme preparations do contain Fe, which not only persists in most of the purification stages, but also increases in proportion as purification proceeds. In this case, therefore, a more detailed examination was necessary, in order to ascertain if Fe was an essential component of the enzyme. It will be shown later that adsorption on tricalcium phosphate gel, and subsequent elution, completely eliminates all Fe from the enzyme, the activity of which is thereby enhanced. Fe is thus a non-essential constituent so far as enzyme activity is concerned.

Cu, on the other hand, is consistently present in all the purified enzyme preparations, which become richer in this element with each step in purification. A study, therefore, of the relation between the Cu content and the activity of purified tea-oxidase preparations would throw some light on the active group of the enzyme. The experiments described in the present communication provide evidence for the postulate that tea oxidase is a Cu-protein complex.

EXPERIMENTAL

As far as possible, particular care was taken to avoid any metallic contamination during preparation of enzyme and in the subsequent experiments. The use of sand in grinding leaf under acetone, as practised previously, was dispensed with in the present investigation. Cu-free distilled water was prepared by distilling ordinary distilled water successively with $KMnO_4$ and metaphosphoric acid in an all-glass apparatus. In the estimation of Cu and Fe, necessary corrections were applied for the small respective metallic contents of reagents.

Estimation of Fe *and* Cu

Fe was estimated by the α-α′-dipyridyl method of Shorland & Wall [1936]. Digestions were carried out in 6 in. × 1 in. pyrex test-tubes with 1 ml. of conc. H_2SO_4 (Analar) and a few drops of Analar HNO_3. The digests were made up to 10 ml., 1 ml. of which was mixed in a ½ in. Lovibond cell with 1 ml. of dipyridyl reagent and a trace of solid $Na_2S_2O_4$. The colorimetric determinations were carried out in a Lovibond tintometer, the red units being found proportional to the Fe content of the test material. With Fe solutions of known strengths, a standardization curve can be drawn (Fig. 1), and the Fe content of any unknown material can be calculated from its Lovibond readings by reference to the standard curve.

The Cu content of the preparations was determined by the colorimetric method of Keilin & Mann [1938], with sodium diethyl-dithiocarbamate reagent. Digestion of the material was carried out as above and 5 ml. digests were employed for the test. The CCl_4 solution was made up to 10 ml. and dehydrated by shaking with a small quantity

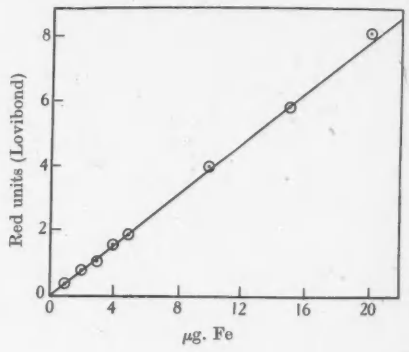

Fig. 1.

of anhydrous sodium sulphate. Colorimetric determination was again carried out in a Lovibond tintometer, with ½ in. cells for all tests. The yellow readings are found to be proportional to the Cu content of the material as shown by Fig. 2, which was obtained by employing solutions of known Cu content. Measurements could be made correct to 0·1 yellow Lovibond units, corresponding to about 0·3 μg. of Cu in 10 ml. of CCl_4 test solution.

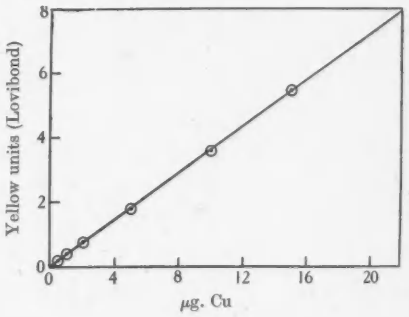

Fig. 2.

Total blanks were run for both the Fe and Cu contents of all reagents except the test solution, the procedure being identical with that of an experiment.

Estimation of enzyme activity

The activities of enzyme preparations were estimated by the method described in Part 4 of this series. Activities are expressed in terms of mg. ascorbic acid oxidized in 1 hr. at room temperature under the conditions described. Where necessary, activities are calculated/g. dry weight of the material.

Purification of enzyme

(*a*) *Extraction of enzyme from acetone-prepared leaf powder.* The acetone preparation of enzyme was obtained as described previously [Lamb & Sreerangachar, 1940] but without using sand for grinding leaf. It was observed previously that extraction of soluble enzyme from this acetone preparation varied with the reaction of the extracting medium up to pH 7·0. Alkaline reactions were not then tried. In the present experiment it has been found that the amount of enzyme extracted increases up to pH 10·0, beyond which the enzyme undergoes considerable inactivation. Sörensen's glycine buffer and Ringer's phosphate buffer were employed to obtain these alkaline reactions, and Table 1 gives results of extraction of 20 mg. acetone-prepared enzyme. After extraction the enzyme solution was adjusted to pH 5·0 with dilute acetic acid, and a fraction equivalent to 20 mg. of original powder was employed for activity determinations. All reactions were carried out in presence of McIlvaine's buffer at pH 5·0 and activity expressed in mg. ascorbic acid oxidized in 1 hr.

Table 1. *Enzyme extraction in alkaline solution*

pH ...	5·0	8·0	9·0	10·0	11·0	12·0
Insoluble enzyme activity	1·51	1·31	1·35	1·05	0·62	0·53
Soluble enzyme activity	0·98	1·02	1·38	1·56	0·32	0·05
Total activity	2·49	2·33	2·73	2·61	0·94	0·58

The unextracted powder gave an average activity of 2·60, which shows that up to pH 10 there is no enzyme inactivation. The amount of enzyme extracted is maximum (60 % of total) at pH 10·0, beyond which both the soluble and insoluble enzymes are considerably inactivated. It appears, therefore, that the most suitable reaction for extraction of soluble enzyme is pH 10·0, which was accordingly employed in the present investigation.

About 10 g. of the acetone-prepared leaf powder were extracted with 400 ml. buffer at pH 10·0 for 2 hr., filtered through muslin and squeezed by hand. The residue was extracted twice with 200 ml. buffer, the extract being squeezed out each time through muslin. The extracts were all collected, filtered through paper to give a clear reddish brown extract and adjusted to pH 6·0–6·5 by addition of dilute acetic acid. This acetone preparation had an activity of 0·21/mg. dry weight, while the activity of its extract was 0·43. If we define the amount of enzyme that oxidizes 1 mg. ascorbic acid as equivalent to a unit of enzyme, the crude extract was found to contain 966 units out of 2110 units of enzyme in the powder, i.e. about 46 % extraction.

(*b*) *Fractional precipitation with ammonium sulphate.* Solid ammonium sulphate was gradually added to the crude extract so as to give, as shown below, increasing concentrations of the salt. The precipitate appearing at each stage was filtered off before the next addition of $(NH_4)_2SO_4$.

Concentration of $(NH_4)_2SO_4$ (% saturation)	Resulting precipitate	
5	No ppt.	
10	No ppt.	
25	Turbidity but no ppt.	
33·3	Slight brown ppt.	(A)
50·0	Thin dark ppt.	(B)
75·0	Dark ppt.	(C)
100·0	Dark ppt.	(D)

Precipitates A–D were collected separately, suspended in water and dialyzed against distilled water until free of $(NH_4)_2SO_4$. They were all found to be completely tannin-free when tested with $FeCl_3$. The precipitates A and B, on dialysis, gave cloudy suspensions containing some insoluble matter, while C gave a brownish solution with very little insoluble substance. Precipitate D, on the other hand, yielded on dialysis a dark brown solution. Table 2 shows the activities and other particulars of these four fractions.

Table 2. *Properties of fractions obtained by* $(NH_4)_2SO_4$ *precipitation*

Fraction	Vol. of suspension (ml.)	Solid content (mg./ml.)	Activity (units/mg. dry wt.)	Enzyme content (units)
A	50	1·80	1·20	105
B	100	1·76	1·31	231
C	30	0·53	5·70	91
D	100	0·88	4·48	394

It will be seen that from an initial quantity of about 966 units a total of about 821 units of enzyme are recovered in the process. The fractions A and B seem to be similar in their activities and also in their solid-matter content. Most of the activity in these fractions appears to be associated with the insoluble matter; for the filtrates from A and B gave activities of only 0·42 and 0·41 unit/ml. respectively, while the original suspensions had activities of 2·17 and 2·31 units/ml. respectively. The dialysates resulting from fractions C and D had much higher activities than A and B, and they were, therefore, mixed and employed for further purification.

(*c*) *Purification with lead acetate.* In accordance with the procedure of Keilin & Mann [1938] for the purification of mushroom polyphenol oxidase, further purification of tea oxidase by means of lead acetate was attempted. In order to determine the amount of lead acetate required to remove impurities without affecting the enzyme, a trial experiment was conducted. 5 ml. portions of the dialysates (§ (*b*) above) were treated separately with 0·1, 0·2, 0·3 and 0·4 ml. 1/100 saturated lead acetate and the resulting

brownish gelatinous precipitate was immediately centrifuged off. The colour of the liquid diminished proportionately with the amount of lead acetate added, and in the last instance the centrifugate was almost colourless. The activities of the various solutions resulting from the above treatment are shown in Table 3.

Table 3. *Activity of material obtained by lead acetate fractionation*

Lead acetate solution added (ml.)	Activity of resulting solution (units/ml.)	Percentage of the original activity recovered
0	2·90	100·0
0·1	2·80	96·5
0·2	2·28	78·6
0·3	0·64	22·0
0·4	0·36	12·4

This preliminary test shows that lead acetate, even in small quantities, brings about a rapid inactivation of enzyme. On the dry weight basis, the activity of the solution resulting from the addition of 0·2 ml. lead acetate solution was 2·85 units/mg., which was only 70% of original activity. The method was, in consequence, useless for the purification of tea oxidase and was abandoned.

(*d*) *Fractional precipitation with acetone in the cold.* 40 ml. enzyme solution (§ (*b*) above) were cooled to −2 to −3° and mixed with cold acetone to give successively the following concentrations of acetone: (1) 5% acetone as employed by Keilin & Mann [1938] to remove readily precipitable impurities; (2) 40% acetone similar to Kubowitz's method [1938] for precipitating the potato oxidase, and (3) >80% acetone which effected more or less total precipitation. After good stirring the brown precipitates were centrifuged off, dissolved in a small quantity of McIlvaine's buffer at pH 7·0 and dialyzed against water.

Table 4. *Properties of the material obtained by fractional precipitation with cold acetone*

Conc. of acetone (%)	Vol. of fraction on dialysis (ml.)	Activity (units/ml.)	Dry matter (mg./ml.)	Activity (units/mg. dry wt.)
5	No ppt.	—	—	—
40	11	13·2	2·8	4·7
>80	9	6·4	2·3	2·8

The activity of the original solution is 5·0 units/mg. dry weight, and this value is not reached by any of the fractions obtained, although the 40% acetone fraction is very near to it. At 40% acetone concentration, therefore, the enzyme is concentrated into a smaller volume, but on the dry-weight basis no purification of enzyme has been achieved. Concentrations of acetone above 40% appear to bring about inactivation of enzyme. Thus acetone precipitation gave no more successful results in the purification of tea oxidase than the precipitation with lead acetate. (Table 4.)

(*e*) *Adsorption on calcium orthophosphate.* Calcium orthophosphate gel has been successfully employed in the purification of several oxidizing enzymes, e.g. mushroom polyphenol oxidase [Keilin & Mann, 1938], laccase [Keilin & Mann, 1938], and tyrosinase [Venkateswaran & Sreenivasaya, 1940]. Preliminary experiments on the use of this material for purification of tea oxidase gave promising results, and it has, therefore, since been adopted. In a typical adsorption experiment 150 ml. of $(NH_4)_2SO_4$-precipitated enzyme, containing 842 units, were acidified to pH 6·5 and mixed with 1·8 g. of freshly prepared gelatinous tricalcium phosphate. After standing for 30 min. the mixture was centrifuged and the colourless centrifugate, which contained only 42 units, was discarded. The phosphate cake was extracted three times with 10 ml. M/2 K_2HPO_4 solution, elution being carried out for $\frac{1}{2}$, 2 and 4 hr. respectively. The mixture was centrifuged once again, and the eluates all combined and dialyzed against distilled water. 120 ml. of pale yellow solution resulted, with 0·53 mg. dry weight/ml. and an activity of 5·0 units/mg. dry weight.

A second adsorption as above resulted in 30 ml. of an almost colourless dialyzed solution: dry weight, 0·20 mg./ml. and activity 10·1/mg. dry weight. A 60–70-fold purification of enzyme in the acetone preparation (activity 0·15 unit/mg. dry weight) has thus been effected by $(NH_4)_2SO_4$ fractional saturation and the two adsorptions just described. This, taken in conjunction with the fact that the soluble enzyme forms, under the most favourable conditions, only 60% of the total quantity of enzyme present in the acetone preparation, shows that the actual concentration of soluble enzyme effected is about 100-fold. Further, on an average the acetone-prepared enzyme forms about 50% of the dry weight of leaf and the latter roughly amounts to a fourth of the fresh weight. These data, therefore, indicate that the activity of the purest preparation obtained is at least 800 times greater than the activity of fresh tea leaf.

There are indications that the stability of the enzyme suffers considerably on purification by adsorption, elution and subsequent dialysis. Most of the inactivation occurs during dialysis which, however, is necessary for the dry-weight determinations and therefore cannot be excluded. It is, therefore, advisable to neutralize the eluate and determine its activity before dialysis. Dry-weight determinations could be done on the dialyzed preparations, with proper allowance for the increase in volume during dialysis. Inactivation can be much reduced if the alkaline eluate is neutralized before dialysis. Thus it is possible that the protein carrier of the enzyme in a purer state cannot stand continuous contact with the alkaline medium, which may rapidly denature the protein. The possibility also of the existence of a protecting substance or an additional prosthetic group which is removed during adsorption cannot be excluded. The work of Keilin & Mann [1940] on the blue pigment of laccase lends support to the latter hypothesis.

Relationship between iron and copper contents and enzyme activity

In order to elucidate any possible relationship between Cu and Fe on the one hand and the activity of the enzyme on the other, quantitative data were obtained on the contents of these metals in the several purified preparations of enzyme as described above. Table 5 contains some typical results of these determinations and also the activities of the preparations/g. dry enzyme. To facilitate comparison, contents of Fe and Cu/unit activity of enzyme are also given.

Table 5. *Relationship between* Fe *and* Cu *contents and enzyme activity*

No.	Stage of purification	Activity (units/g. dry enzyme)	Cu content (μg./g.)	Cu content (μg./unit activity)	Fe content (μg./g.)	Fe content (μg./unit activity)
1	Acetone-prepared enzyme	151	32	0·21	150	1·00
2	Crude extract	330	60	0·18	119	0·36
3	$(NH_4)_2SO_4$ ½ sat. ppt.	890	145	0·16	1364	1·53
4	$(NH_4)_2SO_4$ full sat. ppt. I	3,100	267	0·09	2400	0·77
5	After adsorption on and elution from $Ca_3(PO_4)_2$ gel. I	5,060	625	0·12	0	0
6	$(NH_4)_2SO_4$ full sat. ppt. II	6,600	520	0·08	1558	0·24
7	$Ca_3(PO_4)_2$ adsorbed and eluted enzyme II	4,000	364	0·09	0	0
8	$(NH_4)_2SO_4$ full sat. ppt. III	3,546	335	0·09	789	0·22
9	$Ca_3(PO_4)_2$ adsorbed and eluted enzyme III, first adsorption	5,000	357	0·07	0	0
10	$Ca_3(PO_4)_2$ adsorbed and eluted enzyme III, second adsorption	10,100	800	0·08	0	0
11	Adsorbed cake residue from no. 9 eluted again with alkaline phosphate	2,830	313	0·11	0	0
12	Cake from no. 11 eluted with ½ sat. $(NH_4)_2SO_4$	2,440	244	0·10	610	0·25
13	$(NH_4)_2SO_4$ full sat. enzyme precipitated at 40% acetone concentration	4,700	714	0·15	1786	0·38

It will be noticed that Fe is present in the enzyme preparations up to a certain stage in purification but disappears as purification proceeds further. It actually increases during the initial stages, although this increase is not in proportion to the increase in activity. Absence of Fe in the adsorption-purified enzyme makes its association with the active molecule of the enzyme impossible.

On the other hand, there is a steady increase in the Cu content of the preparations, with increasing activity/g. dry weight of material. In the more active preparations, however, there exists a fair proportionality between enzyme activity and Cu content. The Cu content/unit activity approaches a constant value on progressive purification. When the activities of the purified enzyme preparations are plotted against their corresponding copper contents an approximate straight-line relationship becomes evident (Fig. 3).

There are two points which diverge very appreciably from the linear relationship between activity and Cu content shown by the rest of the data. Against these points the number of the preparation to which they refer is shown. No. 5 is a preparation in which the activity was determined after dialysis following adsorption and elution, in contradistinction to all the rest of the adsorption experiments, where enzyme activity was determined immediately after elution and neutralization. § 3 (*e*) above gives reasons for suspecting that the continued contact with alkaline phosphate inactivates the enzyme in its purer state, and for this reason the low value of enzyme activity here shown is also suspect. No. 13 refers to a fractional precipitation of the enzyme solution with 40% acetone. It is not unreasonable to suggest that the acetone effected both a concentration of the enzyme (which would give a value much higher than the original 5·0) and also inactivated a portion of it. In this case the apparent drop to 4·7 would not represent the full extent of inactivation, and again, this result is rendered suspect. In both these cases, therefore, there are

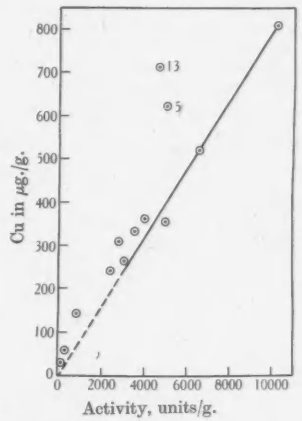

Fig. 3. Relationship between the Cu content and the activity of tea oxidase.

reasonable criteria for rejection of these two data when the activity-Cu relationship is considered.

The proportional relationship between the Cu content and the activity of the preparations is apparent even amongst the batches of acetone-prepared enzyme when such preparations are obtained from individual bushes whose leaf showed a fairly wide range of activity. For instance, the bushes whose acetone-prepared enzyme preparations had activities of 224, 193, 185 and 80 respectively/g. of material contained 20, 16, 16 and 4 μg. of Cu/g. respectively. Since even in these preparations, which can by no means be considered pure, the ratio between the enzyme activities and Cu appears to be of the same order as that obtained with further purified preparations, it is possible that the Cu found in these preparations is mostly enzymic. This idea receives support from the fact that some of the fractions exhibiting only slight activity do also contain some Cu, but it is not in any great excess over the amount that corresponds to their activity. However, not all the Cu present in the fresh leaf belongs to the enzyme. Part of the Cu present in fresh leaf but not essential to the enzyme is indeed removed during extraction with acetone.

Dialysis against KCN

While ordinary dialysis removes all 'free' or ionic Cu from the enzyme preparations, dialysis against cyanide results also in loss of bound Cu from the enzyme. This method of removal of bound Cu was originally devised by Kubowitz [1937; 1938] in his study of potato oxidase and later employed by others [Meiklejohn & Stewart, 1941; Allen & Bodine, 1941]. In the Cu-protein systems studied, complete removal of Cu resulted in total inactivation of enzyme, which showed that Cu formed part of the enzyme molecule.

In experiments with tea-oxidase preparations, dialysis against KCN has also been carried out, with a similar object. To 10 ml. samples of (*a*) $(NH_4)_2SO_4$-purified enzyme and (*b*) $Ca_3(PO_4)_2$-adsorbed enzyme, solid KCN was added to give a concentration of M/100 KCN, and the mixtures dialyzed against Cu-free water until all KCN was removed. In Exps. 2 and 3, the enzyme solutions were first dialyzed for about 4 hr. against M/1000 KCN solution and then against Cu-free water. Table 6 gives the activities and iron and copper contents before and after KCN treatment.

In the case of $(NH_4)_2SO_4$-purified enzyme there is apparently a tendency to retain part of the original Cu after treatment with KCN. By continued dialysis against KCN, as in Exp. 2, most of the Cu can, however, be removed from the enzyme solution. The enzyme preparation obtained after adsorption loses its Cu more easily when subjected to KCN dialysis, which renders this preparation completely free from Cu. It is significant that in every case the activity of the KCN-treated preparation is proportional to the amount of Cu retained in the solution; when the entire Cu is removed, the preparation becomes completely inactive in catalyzing the oxidation of polyphenols.

Table 6. *Dialysis of tea oxidase against* KCN

	Original enzyme	KCN-dialyzed enzyme	Percentage remaining after KCN treatment
Exp. 1. $(NH_4)_2SO_4$-purified enzyme			
Dialyzed against water			
Activity/g.	6000	2800	42
Cu, μg./g.	520	206	40
Fe, μg./g.	1558	1647	106
Exp. 2. $(NH_4)_2SO_4$-purified enzyme			
Dialyzed against KCN + water			
Activity/g.	2872	206	7
Cu, μg./g.	335	35	10
Fe, μg./g.	789	701	89
Exp. 3. $Ca_3(PO_4)_2$-adsorbed enzyme			
Dialyzed against KCN + water			
Activity/g.	3562	0	0
Cu, μg./g.	313	0	0
Fe, μg./g.	0	0	0

The Fe content is not significantly affected by the KCN treatment, and its presence seems to be immaterial for the activity of the preparations.

Previous workers, already cited, have found it possible, by addition of inorganic Cu, to restore the activity of preparations inactivated by dialysis against KCN. Kubowitz [1937; 1938] reports a restoration of 93%, Meiklejohn & Stewart 67%. The behaviour of tea oxidase, however, seems to be different in this respect from the above instances. For, when Cu is removed from tea oxidase by KCN dialysis, it has not been possible to restore activity even after addition of six to ten times the original amount of Cu. For this purpose the inactive dialyzed preparations were allowed, after addition of $CuSO_4$, to stand for a few hours and were then dialyzed against water to remove excess Cu. The preparations remained inactive.

The failure of inorganic Cu to restore the enzyme activity may perhaps be due to its inability, after once being split off, to recombine with the protein residue of the enzyme molecule. The possibly labile nature of the protein part has already been mentioned, and during KCN dialysis it is likely that the protein may have been altered in a manner which makes reconstruction of enzyme impossible. Even in Meiklejohn & Stewart's [1941] experiments, the necessity for adding large excesses of inorganic Cu to restore only part of the original activity may be considered as indicative of the unstable nature of

the protein residue of the enzyme. Although restoration of inactive tea-oxidase preparations has not been successful, the complete loss of activity that attends a total removal of Cu must be regarded as providing evidence for Cu forming the active group of the enzyme.

N *content of active preparations*

Since the purest enzyme is considered to be a Cu protein, its N content may be expected to be about 15–16 % of dry weight, as in the case of other proteins. N determinations of partially purified preparations may, therefore, be taken to indicate the extent of purification effected, provided the preparations do not contain any nitrogenous impurities. It seems unlikely that, after $(NH_4)_2SO_4$ precipitation and dialysis and the operations preceding it, the tea-oxidase preparations contain any unknown nitrogenous impurities. Table 7 gives the results of micro-determinations of N on some of the more active preparations of enzyme, together with their activities.

Table 7. N *contents of enzyme preparations*

Preparations	Activity (units/g. dry wt.)	N (%)
1. $(NH_4)_2SO_4$-precipitated enzyme I	4,700	3·96
2. $(NH_4)_2SO_4$-precipitated enzyme II	3,546	3·47
3. No. 2 after adsorption on $Ca_3(PO_4)_2$	5,000	4·97
4. No. 3 after a second adsorption	10,100	6·60
5. No. 3 elution with $\frac{1}{3}$ sat. $(NH_4)_2SO_4$	2,882	2·73

It will be noticed that the N content of the preparations varies with the degree of purification obtained. If the N content of the pure substance is about 15 %, our best preparation is only about 40 % pure.

DISCUSSION

In plant extracts, a certain amount of catalytic oxidation of ascorbic acid is probably due to the presence of ionic Cu, as distinct from the action of enzymic Cu, which is no doubt very much greater than the former. Since, in the present investigation, estimations of polyphenol oxidase activity have been carried out by means of ascorbic acid, it is relevant to consider if there has been any interference in the results from this source. Such interference, if any, is possible only in the case of acetone-prepared enzyme powder and the crude extract. All other preparations clearly cannot contain any ionic Cu, as they have all been subjected to a prolonged dialysis during the course of purification. The more important conclusions reached in these experiments have been based on the dialyzed preparations, and as such they are unaffected by any possible presence of Cu ions in the fresh leaf.

No correlation has been perceived between the Fe content and the activity of preparations studied. On the contrary, the most active preparations are characterized by a complete absence of Fe, while the Fe contents in other preparations rendered inactive by dialysis against KCN remained unaffected. The possibility, therefore, of the enzyme being of the nature of a haemochromogen is excluded. This is in support of our previous conclusions based on spectroscopic and other evidence.

On the other hand, the evidence presented in this investigation shows a close relationship between the Cu content and tea-oxidase activity. In the more concentrated preparations the catalytic activity runs fairly parallel to the Cu content. Removal of Cu from the enzyme complex by dialysis against KCN results in its inactivation. These results point to the conclusion that, like other known polyphenol oxidases, tea oxidase also is a metalloprotein with Cu as its prosthetic group.

To obtain an idea of the degree of success in the purification of the tea enzyme in our best preparation, a comparison may be made between the activities of our enzyme and that of Kubowitz. Calculated in terms of O_2 uptake, the purest preparation of tea oxidase obtained in these experiments has an activity of 27 μl. O_2/min./μg. of enzymic Cu ($Q_{O_2}/Cu = 1{\cdot}6 \times 10^6$), which is roughly one-tenth the figure obtained by Kubowitz for the potato oxidase. With the limited equipment available here, attempts to purify the enzyme further have not as yet proved successful.

SUMMARY

1. Tea oxidase has been purified to a concentration of at least 800 times that present in fresh leaf.

2. The purest preparation obtained had an activity of 10,100 units/hr./g. dry weight of enzyme, 1 unit of enzyme being defined as the amount which brings about the oxidation of 1 mg. ascorbic acid. The Cu content of this preparation is 0·08 % and nitrogen 6·60 %. It has been calculated that 1 μg. of enzymic Cu catalyzes an O_2 uptake of 27 μl. O_2/min.

3. The enzyme preparations purified by adsorption on $Ca_3(PO_4)_2$ gel were completely free from Fe.

4. In the more active preparations the polyphenol oxidase activity is proportional to the amount of Cu present. Complete removal of Cu inactivates the enzyme; resynthesis of enzyme by addition of $CuSO_4$ has not been possible.

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REFERENCES

Allen, T. H. & Bodine, J. H. [1941]. *Science*, **94**, 443.
Bertrand, G. & Mallèvre, A. [1895]. *C.R. Acad. Sci., Paris*, **121**, 726.
Keilin, O. & Mann, T. [1938]. *Proc. roy. Soc.* B, **125**, 187.
—— —— [1939]. *Nature, Lond.*, **143**, 23.
—— —— [1940]. *Nature, Lond.*, **145**, 304.
Kubowitz, F. [1937]. *Biochem. Z.* **292**, 221.
—— [1938]. *Biochem. Z.* **299**, 32.
Lamb, J. & Sreerangachar, H. B. [1940]. *Biochem. J.* **34**, 1472.
Meiklejohn, G. T. & Stewart, C. P. [1941]. *Biochem. J.* **35**, 755.
Shaw, W. S. & Jones, K. B. W. [1935]. *Uni. Plant. Ass. South India Bull.* **4***b*, 23.
Shorland, F. B. & Wall, E. M. [1936]. *Biochem. J.* **30**, 1047.
Sreerangachar, H. B. [1943]. *Biochem. J.* **37**, 653, 656, 661.
Venkateswaran, S. L. & Sreenivasaya, M. [1940]. *Curr. Sci.* **9**, 21.

Oxidation of Acetic Acid in Animal Tissue

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Although it is known that acetic acid is readily oxidized in the animal, little is known of the intermediate stages on its oxidative path in the animal body, and numerous schemes have been suggested for its intermediate metabolism. In the present paper the oxidation of acetic acid and related 2-carbon compounds in animal tissue was examined. The results suggest that neither glycollic acid, glyoxylic acid, nor glycine are intermediates in the oxidation of acetic acid.

In the course of the investigation it was found that glyoxylic acid is a strong inhibitor of tissue respiration and of carboxylase. This observation may be useful in the study of enzymic reactions.

METHODS

Preparation of glyoxylic acid [Mohrschulz, 1926]. The Ca-salt was isolated and recrystallized three times (analysis: Ca, found 17·9%; calc. 17·9%). The Na-salt used was prepared from the Ca-salt by an equimolecular quantity of Na_2CO_3.

Manometric experiments were carried out at 40° in Warburg's conical flasks. Fresh tissue was either sliced or minced. In every experiment tissue from the same normally fed animal was used, and the results are therefore directly comparable.

The rate of disappearance of carboxylic acids was studied by a method first described by Meyerhof & Lohmann [1926] [see also Elliott & Schroeder, 1934]. The method is based on the fact that the oxidation of a carboxylic acid in a neutral buffered medium yields, under certain conditions, bicarbonate. The increase of bicarbonate in the solution is therefore a measure of the oxidation of the organic acid. Tissue slices (dry weight 7–30 mg. according to species and tissue) were suspended in 3 ml. physiological saline [Krebs & Henseleit, 1932] containing 80μl. bicarbonate/ml. The substrates were added as Na-salts to the main compartment of the flask. 0·2 ml. 2N NaOH was placed into the centre well and, unless otherwise stated, the gas space of the cup was filled with O_2. The manometers were put into the thermostat and the O_2 uptake was measured after an equilibration period of 10 min. The total O_2 uptake was calculated by extrapolating for the equilibration period. After the incubation period (90–120 min.) the cups were cooled with ice, and the slices removed, washed, dried, and weighed. The NaOH in the centre well was replaced by 0·1 ml. 10% H_2SO_4, and 0·2 ml. 10% H_2SO_4 was measured into the side-arm. The gas space was filled with 5% CO_2 in O_2, and, after equilibration, the amount of $NaHCO_3$ in the solutions was estimated by acidification. The values found were corrected for the amount of $NaHCO_3$ carried by the tissue slices, on the assumption that a volume of liquid equalling 10 times the dry weight of tissue is introduced or removed with the tissue. From the values obtained, Q_{O_2} and $Q_{\text{bicarbonate}}$ ($Q_{\text{bic.}}$) were calculated. $Q_{\text{bic.}}$ is equal to the negative metabolism quotient of the substrate investigated ($-Q_{\text{substrate}}$), provided that no acid groups are formed during the experiment, and the carboxylic group is oxidized. When the carboxylic group is not altered as a result of the metabolism, the changes of bicarbonate found in the experiment are not related to the utilization of the substrate.

In experiments with minced tissue, 3 ml. of a suspension of tissue in phosphate saline [Krebs, 1933] were measured into the main compartment; 0·2 ml. 2N NaOH was placed into the centre well, and the substrate was measured into the side-arm and made up to 1 ml. with the saline medium. The gas space was filled with O_2. After an equilibration period of 10 min. the contents of the cup were mixed and the O_2 uptake was measured.

In anaerobic experiments, yellow phosphorus was placed in the centre well, the gas space filled with 5% CO_2 in N_2, and the CO_2 output measured.

In some of the experiments lactic acid was estimated according to Friedemann, Cotonio & Shaffer [1927].

RESULTS

Oxidation of acetate. As measured by the formation of bicarbonate, this is more rapid in guinea-pig kidney cortex than in any other tissue examined

(see Table 1): 0·0156 M acetate (final concentration) increases Q_{O_2} about 50 % above that of a control without substrate, and the average $Q_{bic.}$ is +7·6 as compared with +1·0 in absence of any substrate (average of 16 experiments). Elliott & Schroeder [1934] and Elliott, Benoy & Baker [1935] found in the presence of acetate a $Q_{bic.}$ of 4·7 in rabbit and 4·1 in rat kidney. $Q_{bic.}$ in the presence of acetate increases with the concentration of substrate (Table 2).

Table 1. *Oxidation of acetate in various tissues*

Tissue	Acetate added (M)	Q_{O_2}	$Q_{bic.}$
Guinea-pig: Kidney	0	−12·5	+2·0
	0·0156	−18·1	+7·3
Liver	0	− 6·4	+0·28
	0·0156	− 7·6	+2·27
Brain	0	− 8·0	+0·56
	0·0156	− 7·5	+1·15
Rat: Kidney	0	−23·2	+1·12
	0·0156	−29·6	+4·2

Table 2. *Effect of substrate concentration on the oxidation of acetate in guinea-pig kidney cortex*

Acetate conc. (M)	Q_{O_2}	$Q_{bic.}$
0	− 9·8	+0·68
0·00125	−17·2	+3·1
0·0025	−17·3	+4·0
0·005	−18·4	+5·5
0·01	−20·0	+6·4
0·02	−21·6	+7·2

Effect of pH. The effect of pH on acetate oxidation was studied by using gas mixtures containing different concentrations of CO_2 for filling the vessels. No NaOH was placed into the centre well. A higher rate of $NaHCO_3$ formation from acetate was found when the manometers were filled with a mixture of CO_2 and O_2, instead of O_2, with NaOH in the centre well. $Q_{bic.}$ slightly decreases with increasing concentrations of CO_2 in the gas mixture (Table 3).

Table 3. *Effect of* pH *on acetate oxidation*

Gas mixture	Solution in centre well	Approx. pH (calc.)	Acetate added (M)	Q_{O_2}	$Q_{bic.}$
O_2	NaOH	7·33	0	−12·2	+1·28
O_2	NaOH	7·33	0·015	−17·7	+5·91
1 % CO_2 in O_2	None	7·33	0·015	—	+7·8
5 % CO_2 in O_2	None	6·6	0·015	—	+6·9
20 % CO_2 in O_2	None	6·0	0·015	—	+6·32

Effect of ammonium ions. Ammonium ions have a catalytic effect on the utilization of some substrates in kidney cortex [Edson, 1935; Krebs & Cohen, 1939]. Table 4 shows that NH_4Cl inhibits the oxidation of acetate, as measured by bicarbonate formation; the O_2 uptake is also reduced. No appreciable disappearance of NH_4^+ was observed. Inhibition of respiration by NH_4Cl has been previously observed in liver [Edson, 1935], in brain [Weil-Malherbe, 1938] and in the presence of acetoacetate in kidney [Edson, 1935].

Table 4. *Effect of* NH_4Cl *on acetate oxidation*

Additions (final concn.)			
Acetate (M)	NH_4Cl (μl.)	Q_{O_2}	$Q_{bic.}$
0	—	−13·5	+1·61
0·0156	—	−22·2	+9·15
0	1120	−13·0	+0·91
0·0156	224	−16·4	+4·35
0·0156	560	−17·5	+5·78
0·0156	1120	−17·2	+3·4
0·0156	2240	−17·6	+2·92

Effect of anaerobic conditions. No bicarbonate is formed from acetate under anaerobic conditions (Table 5). This confirms unpublished experiments of Dr D. Herbert [private communication], made by different methods.

Table 5. *Effect of anaerobic conditions on the bicarbonate formation from acetate*

Acetate added (M)	CO_2 liberated (μl.)	$NaHCO_3$ found (μl.)	Total CO_2 (μl.)
0	41	235	276
0·0156	39	228	267

Effect of malonate. Malonate was found to inhibit the formation of bicarbonate from acetate in guinea-pig kidney cortex (Table 6). The inhibition is slight at a concentration of 0·001 M, whilst almost complete inhibition is found at a final concentration of 0·031 M. This inhibition of acetate oxidation is abolished neither by glutamate nor by glucose. Inhibition of the oxygen uptake of liver slices in the

Table 6. *Effect of malonate on the bicarbonate formation from acetate*

Exp. no.	Additions: Acetate (M)	Additions: Malonate (M)	Q_{O_2}	$Q_{bic.}$
1	—	—	−15·1	+2·18
	0·0156	—	−22·4	+9·1
	0·0156	0·031	−13·5	+1·9
2	—	—	−15·7	0·0
	—	0·01	−13·0	+0·87
	0·0156	—	−23·3	+8·52
	0·0156	0·01	−14·8	+3·7
	0·0156	0·003	−17·2	+6·4
	0·0156	0·001	−17·8	+8·0

presence of acetate has been reported by Jowett & Quastel [1935].

Oxidation of 2-carbon compounds in kidney cortex. Challenger, Subramaniam & Walker [1927] suggested that in moulds acetate is oxidized to form glycollic, glyoxylic, and oxalic acids. This scheme of oxidation has also been suggested for animal tissue [Dakin, 1922; Toeniessen & Brinkmann, 1930]. The oxidation by kidney cortex of glycollic and glyoxylic acids and of two other 2-carbon compounds, glycine and ethanol, which might possibly be expected to be connected with the metabolism of acetate, was tested (Table 7). Neither glycollate, which was shown to be oxidized by rat- and rabbit-liver preparations [Dohan, 1940], nor glycine, is appreciably oxidized by guinea-pig kidney cortex. On the other hand, glyoxylate was found to be a strong inhibitor of tissue respiration. $Q_{bic.}$ of kidney tissue in presence of 0·0156M or lower concentrations of glyoxylate was generally negative. This observation indicates the formation of free acidic groups. Ethanol has no marked effect on the O_2 uptake; this confirms the results of Leloir & Muñoz [1938].

Table 7. *Oxidation of 2-carbon compounds in guinea-pig kidney cortex*

Exp. no.	Substrate	Q_{O_2}	$Q_{bic.}$
1	—	−14·3	+0·85
	0·0156M acetate	−20·4	+6·76
	0·0156M glycollate	−14·7	+0·99
2	—	−13·0	+1·85
	0·0156M acetate	−16·6	+5·15
	0·0156M glycine	−12·6	+1·82
3	—	−13·6	0·0
	0·0156M acetate	−20·2	+5·8
	0·0156M glyoxylate	− 7·2	−3·68
	0·0065M glyoxylate	−13·5	−1·5
	0·0032M glyoxylate	−14·5	−0·44
4	—	−12·2	+1·28
	0·0156M acetate	−17·7	+5·91
	0·0156M ethanol	−12·55	+1·12

Inhibition by glyoxylate. Glyoxylate has a strong inhibitory effect on the respiration of liver, muscle and kidney. In minced pigeon-breast muscle and liver the inhibition of respiration increases with time (Fig. 1; Table 8). The glyoxylate inhibition in pigeon-breast muscle is not abolished by citrate, fumarate or pyruvate, and only slightly by α-ketoglutarate or succinate.

Table 8. *Effect of glyoxylate on the respiration of minced pigeon-breast muscle and liver*

Cup	Tissue	Tissue wet wt. (mg.)	Glyoxylate (M)	O_2 uptake/ 60 min. (μl.)
1	Muscle	250	0	1179
2	Muscle	250	0·00625	441
3	Liver	400	0	865
4	Liver	400	0·00625	443

Under anaerobic conditions, acid or CO_2 production of guinea-pig kidney cortex and of liver is increased by the presence of glyoxylate. No increased formation of lactic acid in the presence of glyoxylate was observed in liver tissue (Table 9). Glyoxylate therefore does not increase the glycolysis of liver, and it may be assumed that the formation of free acidic groups in the presence of glyoxylate under anaerobic conditions is due to a coupled oxido-reduction of the substrate according to the reaction:

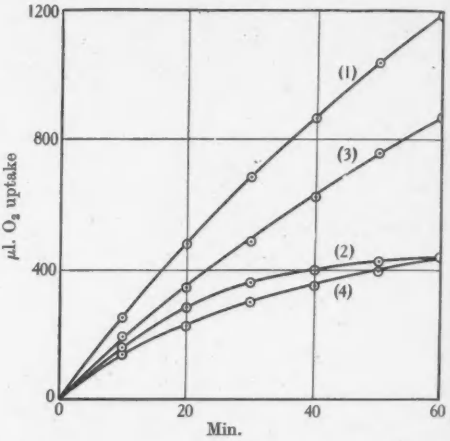

Fig. 1. The effect of 0·00625M glyoxylate on the respiration of minced pigeon-breast muscle and liver. The numbers of the curves correspond with the numbers of cups in Table 8.

$$2COOH.CHO + H_2O = COOH.COOH + COOH.CH_2OH.$$

Dismutation of glyoxylic acid according to the above reaction has been reported to occur in the presence of strong alkali [Böttinger, 1880], or of yeast maceration juice [Stepanow & Kusin, 1930].

Table 9. *Effect of glyoxylate on formation of* CO_2 *and lactic acid under anaerobic conditions*

No.	Tissue (of guinea-pig)	Glyoxylate (M)	Q_{CO_2}	Q_{lactic}
1	Kidney	0	1·39	—
		0·0143	3·13	—
	Liver	0	1·85	—
		0·0143	3·0	—
2	Liver	0	3·56	3·87
		0·0143	5·30	3·83

Effect of glyoxylate on carboxylase. The decarboxylation of pyruvate by fresh yeast maceration juice is inhibited by glyoxylate. This inhibition

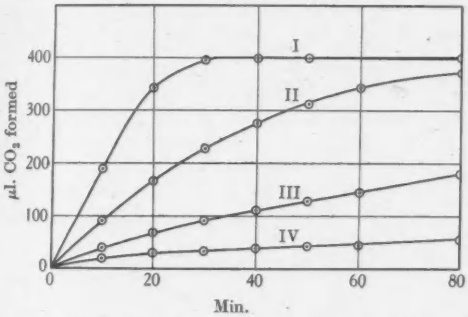

Fig. 2. Effect of glyoxylate on the decarboxylation of pyruvate by yeast maceration juice. Cup content: 400μl. pyruvate, 0·3 ml. 3M acetate buffer pH 5·0, 3·5 ml. H_2O. Side-arm: 1 ml. yeast maceration juice. Volume made to 5 ml., temp. 25°. Conc. of glyoxylate: I = 0; II = 0·001M; III = 0·002M; IV = 0·004M.

increases with increasing concentrations of glyoxylate (Fig. 2). It is probable that glyoxylate competes with pyruvate for the active groups of carboxylase.

DISCUSSION

The investigation of the oxidation of glycine, glycollic and glyoxylic acids in guinea-pig kidney cortex, as measured by the formation of bicarbonate, has shown that none of these substrates can be an intermediate in the oxidative path of acetate. Apart from the substrates investigated, the only substance that could arise by direct oxidation of acetic acid is oxalic acid, which is known to be little utilized in animal tissue [Dakin, 1922]. It seems that the oxidation of acetic acid follows other pathways, probably involving condensation reactions. Condensations of acetic acid have been demonstrated by Loeb [1912], who found that acetoacetic acid is formed from acetic acid, and recently Slade & Werkman [1942], using isotopic carbon, demonstrated the condensation of acetic acid to succinic acid and butylene glycol in bacteria. Lipmann [1941] suggests that acetate condenses with phosphate to form acetylphosphate before undergoing oxidation.

SUMMARY

1. The oxidation of acetate, glycollate, and glyoxylate in guinea-pig kidney cortex was studied by examining the effect of these substrates on the O_2 uptake and the formation of bicarbonate.

2. The oxidation of acetate, as measured by the formation of bicarbonate, was more rapid in guinea-pig kidney cortex than in any other tissue examined. On the average, 0·0156M acetate raised the Q_{O_2} by about 50% and $Q_{bic.}$ was +7·6 as compared with a $Q_{bic.}$ + 1·0 in absence of added substrate.

3. The oxidation of acetate was found to increase with the substrate concentration; it depends on the pH, the highest $Q_{bic.}$ having been found when the pH of the medium was decreased by filling the vessel with 1% CO_2 in O_2; NH_4^+ decreased the oxidation of acetate; no bicarbonate was formed under anaerobic conditions.

4. Malonate inhibited the oxidation of acetate in guinea-pig kidney. The inhibition was almost complete at a concentration of 0·031M malonate.

5. Glycine, glycollic acid, and ethanol were not appreciably oxidized in guinea-pig kidney. Glyoxylic acid is a strong inhibitor of tissue respiration.

6. Glyoxylic acid strongly inhibits the decarboxylation of pyruvate by fresh yeast maceration juice.

7. Glyoxylic acid to a small extent may undergo a coupled oxido-reduction in liver and kidney tissue to form glycollic and oxalic acids.

8. It is concluded that neither glycollic nor glyoxylic acid is an intermediate in the oxidation of acetic acid in guinea-pig kidney cortex.

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REFERENCES

Böttinger, C. [1880]. *Ber. dtsch. chem. Ges.* **13**, 1932.
Challenger, F., Subramaniam, V. & Walker, T. K. [1927]. *J. chem. Soc.* p. 200.
Dakin, H. D. [1922]. *Oxidations and Reductions in the Animal Body*, 2nd ed. London: Longmans, Green.
Dohan, J. S. [1940]. *J. biol. Chem.* **135**, 793.
Edson, N. L. [1935]. *Biochem. J.* **29**, 2082.
Elliott, K. A. C., Benoy, M. P. & Baker, Z. [1935]. *Biochem. J.* **29**, 1937.
—— & Schroeder, E. F. [1934]. *Biochem. J.* **28**, 1920.
Friedemann, T. E., Cotonio, M. & Shaffer, P. A. [1927]. *J. biol. Chem.* **73**, 359.
Jowett, M. & Quastel, J. H. [1935]. *Biochem. J.* **29**, 2181.
Krebs, H. A. [1933]. *Hoppe-Seyl. Z.* **217**, 191.
Krebs, H. A. & Cohen, P. P. [1939]. *Biochem. J.* **33**, 1895.
—— & Henseleit, K. [1932]. *Hoppe-Seyl. Z.* **210**, 33.
Leloir, L. F. & Muñoz, J. M. [1938]. *Biochem. J.* **32**, 299.
Lipmann, F. [1941]. *Advances of Enzymology*, **1**, 99.
Loeb, A. [1912]. *Biochem. Z.* **47**, 118.
Meyerhof, O. & Lohmann, K. [1926]. *Biochem. Z.* **171**, 381.
Mohrschulz, W. [1926]. *Z. Elektrochem.* **32**, 434.
Slade, H. D. & Werkman, C. H. [1942]. *Arch. Biochem.* **2**, 97.
Stepanow, A. & Kusin, A. [1930]. *Ber. dtsch. chem. Ges.* **63**, 1147.
Toeniessen, E. & Brinkmann, E. [1930]. *Hoppe-Seyl. Z.* **187**, 137.
Weil-Malherbe, H. [1938]. *Biochem. J.* **32**, 2257.

The Metabolism of Butyric and Related Acids in Animal Tissue

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Butyric acid is known to undergo a number of metabolic reactions in animal tissue, viz. oxidation to acetoacetic and β-hydroxybutyric acids, formation of carbohydrate [Cross & Holmes, 1937; Blixenkrone-Møller, 1938], and complete oxidation. The intermediate steps in these reactions are, however, still largely unknown. In the present paper experiments on the oxidation in animal tissue of butyric acid and a number of hitherto unexamined 4-carbon monocarboxylic acids will be reported. The metabolism of the latter was studied with the object of ascertaining whether they might be intermediates in the metabolism of butyric acid. It will be shown that vinylacetic, *dl*-$\alpha\gamma$-dihydroxybutyric, γ-hydroxybutyric, and in some tissues *trans*-γ-hydroxycrotonic acids are oxidized in animal tissues; *dl*-$\beta\gamma$-dihydroxybutyric acid is little oxidized in kidney cortex, but causes the formation of a β-ketonic acid in starved rat and pigeon liver; tetrolic and tetronic acids are not oxidized in animal tissues, whilst vinylglycollic acid is an inhibitor of tissue respiration.

EXPERIMENTAL

Preparations

γ-Hydroxybutyric acid was prepared according to Fichter & Hérbrand [1896]. b.p. 202°. The preparation was free from succinic acid.

Vinylacetic acid [Linstead, Noble & Boorman, 1933]. b.p. 72·5–75°/13 mm.; 39·5 mg. required 4·54 ml. 0·1 N NaOH; calc. 4·60 ml.

Tetrolic acid [Feist, 1906]. m.p. 75·5°.

dl-*$\alpha\gamma$-Dihydroxybutyric acid* [Nef, 1910]. The lactone of the acid, together with lactic acid, was extracted with ether from a mixture of saccharinic acids obtained by treating lactose with 8 N NaOH. The acids were distilled at 5–6 mm., b.p. 115–120°; the distillate was neutralized with $NaHCO_3$, and the α-hydroxybutyrolactone was extracted with ether. The extracted lactone was converted into the phenylhydrazide (m.p. 130°), the hydrazide was decomposed with $Ba(OH)_2$, the phenylhydrazine extracted with ether and the lactone extracted after acidifying with H_2SO_4; 42·1 mg. required on heating 4·05 ml. 0·1 N NaOH, calc. 4·04 ml.

dl-*$\beta\gamma$-Dihydroxybutyric acid* [Nef & Dar Juan, 1910]. 89·2 mg. required on heating 4·25 ml. 0·2 N NaOH, calc. 4·37 ml.

trans-*γ-Hydroxycrotonic acid* [Rambaud, 1935]. m.p. 107·5°.

Vinylglycollic acid was isolated from the higher boiling fractions after the distillation of its methyl ester [van der Sleen, 1902]. b.p. 127·5°/11 mm.; 100·8 mg. required on heating 4·90 ml. 0·2 N NaOH, calc. 4·95 ml. An attempt to isolate the crystalline Ba-salt [van der Sleen, 1902] was unsuccessful.

Tetronic acid was prepared by Dr H. A. Krebs according to Wolff & Schwabe [1896].

The other substrates were commercial preparations. The substrates were used as Na-salts.

Methods

1. The experiments were carried out on slices of kidney tissue from well-fed animals, unless it is stated that fasting animals were used. The oxidation of the various substrates was studied by the method of Meyerhof & Lohmann [1926] (see Kleinzeller [1943]). The formation of bicarbonate from crotonic and vinylacetic acids, as measured by this method, was compared with the disappearance of double bonds in the presence of these acids; the two methods were found to give similar results (Tables 6 and 7). As there are no specific methods available for estimating the other substrates, the assumption that the formation of bicarbonate is a measure of the utilization of these substrates is necessarily hypothetical.

2. Acetoacetic acid was estimated by the method of Edson [1935], which is specific for β-ketonic acids.

3. Estimation of crotonic and vinylacetic acids: The method is based on the estimation of H_2 necessary for the quantitative catalytic hydrogenation of the double bond. Both crotonic and vinylacetic acids are quantitatively hydrogenated under the conditions used for the estimation of aconitic acid [Johnson, 1939]. The conical manometric cups were filled as follows:

Main compartment: 2·0 ml. water, 0·5 ml. 5% H_2SO_4, 0·5 ml. catalyst suspension containing about 5 mg. Pd catalyst (Membranfilter Ges. Göttingen, 5% Pd on $BaSO_4$), finely ground and saturated with H_2.

Centre well: 0·1 ml. 20% NaOH, 0·1 ml. 20% pyrogallic acid, small glass bead.

Side-arm: 1 ml. test solution (acidified).

The cups were filled with H_2, and put into a bath at 40°. Equilibrium was quickly attained. Since no H_2 uptake was observed in the blank (without addition of substrate), it could be generally omitted. Recovery of added material was satisfactory (Table 1).

Table 1. *Estimation of crotonic and vinylacetic acids*

Acid added (μl.)	μl. H_2 uptake after 5 min.	10 min.	15 min.	20 min.
448 crotonic	381	447	448	446
*448 vinylacetic	410	421	421	421
*465 vinylacetic	405	450	455	456

* The somewhat low recovery of vinylacetic acid may be due to loss of this volatile acid during preparation of the solution.

In manometric experiments the following procedure was adopted: the contents of the cup were deproteinized with ⅓ vol. of 5% HPO_3 and filtered; a measured portion was then transferred into a Kutscher Steudel extractor, acidified, and extracted with ether. The bulb of the extractor contained 1 ml. M $NaHCO_3$ to neutralize the extracted free acid and thus to prevent losses due to their volatility. The extraction was generally complete after 1 hr. (Table 2). After the extraction the ether was distilled off, and a measured portion of the acidified extract was used for the estimation.

Table 2. *Extraction of crotonic and vinylacetic acids*

(Volume of solution in extractor: 20 ml. Approximate rate of distillation of ether: 15 ml./min.)

Acid added (μl.)	Acid found (μl.) after extraction		
	0–30 min.	30–60 min.	Total
896 crotonic	881	16·5	897·5
834 vinylacetic	830	5	835

The method is not specific, for many unsaturated compounds, and also keto-acids, will be estimated in this way. The disappearance or formation of unsaturated compound will be denoted by the symbol Q_Δ, i.e. μl. H_2 absorbed/mg. dry weight of tissue/hr.

RESULTS

Oxidation of 4-carbon monocarboxylic acids in kidney cortex. It is known that kidney cortex oxidizes butyric, crotonic, β-hydroxybutyric, acetoacetic [Jowett & Quastel, 1935; Quastel & Wheatley, 1935] and *threo*-αβ-dihydroxybutyric acid [Weil-Malherbe, 1938; see, however, Hoff-Jørgensen, 1940].

In the present experiments the oxidation in guinea-pig and rat kidney cortex of various substrates at a concentration of 0·0156 M was tested by means of measurements of the O_2 uptake and the bicarbonate formation; the results are summarized in Tables 3 and 4. It will be seen that butyric, crotonic, vinylacetic, β-hydroxybutyric, γ-hydroxybutyric, and *dl*-αγ-dihydroxybutyric acids are oxidized in guinea-pig and rat kidney cortex. These substrates considerably increase Q_{O_2} and $Q_{bic.}$ above those of a control. *trans*-γ-Hydroxycrotonic acid is oxidized in rat kidney only. The following substrates are slowly oxidized in kidney slices of both species: *dl*-βγ-dihydroxybutyric acid, tetrolic, tetronic, and α-hydroxybutyric acids. Vinylglycollic acid inhibits tissue respiration, and $Q_{bic.}$ was found to be negative. The high rate of oxidation of γ-hydroxybutyric, *dl*-αγ-dihydroxybutyric acid, and in rat kidney *trans*-γ-hydroxycrotonic acid, suggests that the hydroxyl group is oxidized with the formation of succinic, malic, and fumaric acids respectively. The recent observation by Lipmann & Perlmann [1942] that tetrolic acid is not attacked in kidney tissue is confirmed.

A study was then made of the rates of oxidation in rat kidney of butyric and related acids by measurement of Q_{O_2} and $Q_{bic.}$. In further experiments, again using rat kidney, as it oxidizes butyric acid at a higher rate than guinea-pig kidney (see Table 3), the disappearance of crotonic and vinylacetic acids and the formation of unsaturated compounds during the oxidation of butyric acid and β-hydroxybutyric acid were measured. As shown in Table 5, the latter acid is oxidized at a slower rate than the former, confirming the observation of Dakin [1922] on the behaviour of these substrates when administered to dogs. It seems unlikely, therefore, that β-hydroxybutyric acid can be an intermediate on the main pathway of the oxidation of butyric acid in rat kidney slices. It will be noted that vinylacetic acid was oxidized at a rate slightly higher than that of either butyric or

Table 3. *Oxidation of 4-carbon monocarboxylic acids in guinea-pig and rat kidney cortex*

Acid added as substrate (0·0156 M)	Formula	Kidney cortex from Guinea-pig: No. of exps.	Guinea-pig: $Q_{bic.}$	Rat: No. of exps.	Rat: $Q_{bic.}$
None	—	12	0·00–1·97	5	0·10–1·97
Butyric	$CH_3.CH_2.CH_2.COOH$	4	1·53–2·19	5	3·46–6·70
Crotonic	$CH_3.CH:CH.COOH$	4	1·98–3·17	3	2·31–5·59
Vinylacetic	$CH_2:CH.CH_2.COOH$	4	3·13–5·10	3	5·57–9·85
Tetrolic	$CH_3.C\equiv C.COOH$	2	0·40; 1·84	1	−0·9
α-Hydroxybutyric	$CH_3.CH_2.CHOH.COOH$	1	1·77	—	—
β-Hydroxybutyric	$CH_3.CHOH.CH_2.COOH$	3	3·22–4·30	2	4·44; 4·82
γ-Hydroxybutyric	$CH_2OH.CH_2.CH_2.COOH$	7	2·75–4·20	3	5·20–8·10
trans-γ-Hydroxycrotonic	$CH_2OH.CH:CH.COOH$	2	1·21; 0·28	3	5·50–10·0
dl-αγ-Dihydroxybutyric	$CH_2OH.CH_2.CHOH.COOH$	3	2·90–3·78	3	5·70–9·90
dl-βγ-Dihydroxybutyric	$CH_2OH.CHOH.CH_2.COOH$	4	0·30–3·10	1	1·4
Tetronic	$CH_2CO.CH_2.CO$ (O)	2	1·65; 2·43	1	−0·4
Vinylglycollic	$CH_2:CH.CHOH.COOH$	3	−0·35–−2·60	1	−3·2

Table 4. *Oxidation of 4-carbon monocarboxylic acids in kidney cortex*

Exp.	Substrate added (0·0156 M)	Q_{O_2}	$Q_{bic.}$
Guinea-pig			
1	None	−14·3	+ 0·3
	Butyrate	−22·8	+ 1·57
	Crotonate	−22·3	+ 1·98
	γ-Hydroxybutyrate	−18·6	+ 4·2
	trans-γ-Hydroxycrotonate	−18·4	+ 1·2
	dl-βγ-Dihydroxybutyrate	−17·3	+ 3·1
	Vinylglycollate	− 7·2	− 0·35
2	None	−14·0	+ 1·15
	γ-Hydroxybutyrate	−16·6	+ 4·05
	dl-αγ-Dihydroxybutyrate	−14·35	+ 3·78
3	None	−11·2	+ 0·32
	Crotonate	−17·7	+ 2·06
	trans-γ-Hydroxycrotonate	−14·5	+ 0·32
4	None	−14·3	+ 0·32
	α-Hydroxybutyrate	−17·3	+ 1·77
	Tetronate	−14·8	+ 1·65
5	None	−12·2	+ 1·28
	Butyrate	−19·4	+ 2·19
	Tetrolate	−12·6	+ 1·84
6	None	−14·6	+ 0·75
	dl-βγ-Dihydroxybutyrate	−14·9	+ 1·08
	Vinylglycollate	− 5·7	− 2·6
7	None	−12·1	+ 1·67
	Vinylacetate	−22·1	+ 3·13
	dl-αγ-Dihydroxybutyrate	−16·7	+ 2·83
Rat			
8	None	−23·2	+ 1·12
	Butyrate	−32·2	+ 3·9
	α-Hydroxybutyrate	−22·6	—
	γ-Hydroxybutyrate	−27·7	+ 8·15
	trans-γ-Hydroxycrotonate	−25·2	+10·0
	dl-αγ-Dihydroxybutyrate	−25·7	+ 7·52
	dl-βγ-Dihydroxybutyrate	−25·9	+ 1·4
	Tetrolate	−15·6	− 0·9
	Tetronate	−26·5	− 0·4
	Vinylglycollate	− 5·8	− 3·2
9	None	−18·8	+ 1·97
	Butyrate	−29·4	+ 3·46
	Crotonate	−21·3	+ 2·31
	γ-Hydroxybutyrate	−29·9	+ 5·25
	trans-γ-Hydroxycrotonate	−24·6	+ 9·05
	dl-αγ-Dihydroxybutyrate	−27·4	+ 9·9

crotonic acid. Crotonic acid was generally found to be oxidized in rat kidney at a lower rate than butyric acid (see also Exp. 9, Table 4). According to these results vinylacetic acid could possibly be an intermediate in the oxidation of butyric acid in rat kidney but this needs to be ascertained by further investigations.

The rate of disappearance of double bonds in the presence of crotonic and vinylacetic acids is of the same order as the rate of bicarbonate formation from these acids (Tables 5 and 6). Butyric and β-hydroxybutyric acids yield an unsaturated compound, but as the method of estimating such compounds is not specific, no conclusions on the nature of the product formed can be drawn from this observation. Under anaerobic conditions vinylacetic acid disappears in rat kidney; Lipmann & Perlmann [1942] found under similar conditions a disappearance of crotonic acid.

The amount of unsaturated compound found when butyric acid is oxidized in rat kidney does not increase after the first hour, so that Q_{Δ} decreases, as shown in Table 7. As Jowett & Quastel [1935] have already shown, only small amounts of acetoacetic acid are formed in rat kidney in the presence of butyric acid, and the unsaturated compound formed is therefore unlikely to be acetoacetic acid.

Table 5. *Rates of oxidation of butyric and related acids in rat kidney*

No.	Substrate added (0·0156 M)	Q_{O_2}	$Q_{bic.}$	Q_{Δ}
1	None	−22·8	+1·85	—
	Butyrate	−26·5	+6·70	—
	Crotonate	−25·2	+5·49	—
	Vinylacetate	−27·4	+9·85	—
	β-Hydroxybutyrate	−28·8	+4·82	—
2	None	−21·2	+0·12	+0·12
	Butyrate	−29·8	+5·15	+3·13
	Crotonate	−26·0	+3·36	−3·54
	Vinylacetate	−27·4	+5·57	−7·96
	(Vinylacetate; anaerobic	—	—	−2·89)
	β-Hydroxybutyrate	−28·8	+4·44	+1·92

Table 6. *Oxidation of vinylacetic acid in rat kidney*

(705 μl. vinylacetic acid added. The results refer to the whole period of the experiment.)

Duration of exp. (hr.)	Without substrate			With vinylacetate		
	Q_{O_2}	$Q_{bic.}$	Q_{Δ}	Q_{O_2}	$Q_{bic.}$	Q_{Δ}
1	−23·4	+1·88	+0·37	−29·1	+5·79	−6·30
2	−22·2	+1·43	+0·15	−27·7	+5·10	−4·35
4	−23·1	+1·80	+0·04	−27·7	+4·54	−5·45

Table 7. *Oxidation of butyric acid in rat kidney*

(The results refer to the whole period of the experiment.)

Duration of exp. (hr.)	Without substrate			With 0·0156 M butyrate		
	Q_{O_2}	$Q_{bic.}$	Q_{Δ}	Q_{O_2}	$Q_{bic.}$	Q_{Δ}
1	−22·4	+0·39	+0·69	−32·6	+4·66	+6·0
2	—	—	—	−27·6	+4·08	+1·92
4	−20·4	+0·59	+0·26	−27·5	+3·39	+0·56

Effect of malonate. 0·031 M malonate inhibits the oxidation of all the substrates examined in guinea-pig and rat kidney (Table 8). A slight formation of bicarbonate in the presence of malonate was observed in rat kidney when *trans*-γ-hydroxycrotonic acid was the substrate. The inhibition of the oxidation of butyric acid by malonate has already been reported by Jowett & Quastel [1935].

Table 8. *Effect of malonate on the oxidation of 4-carbon monocarboxylic acids in kidney cortex*

Exp. no.	Substrate added (0·0156 M)	Substrate alone Q_{O_2}	Substrate alone $Q_{bic.}$	Substrate +0·031 M malonate Q_{O_2}	Substrate +0·031 M malonate $Q_{bic.}$
Guinea-pig					
1	None	−14·5	0·0	−10·2	+1·1
	Butyrate	−20·0	+2·1	−12·3	+0·9
	β-Hydroxybutyrate	−18·3	+3·3	−12·0	+1·4
	γ-Hydroxybutyrate	−20·0	+3·0	−11·0	+0·3
2	None	−11·4	+0·3	—	—
	Crotonate	−17·7	+2·1	− 9·3	+0·3
	trans-γ-Hydroxy-crotonate	−14·6	+0·3	− 8·5	0·0
	dl-βγ-Dihydroxy-butyrate	−13·7	+0·3	− 9·4	—
3	None	−12·9	+0·86	—	—
	Tetronate	−14·9	+2·43	−10·5	+0·41
	Tetrolate	−13·0	+0·42	− 9·4	+0·22
4	None	−14·3	+1·97	−10·6	+0·74
	Vinylacetate	−22·8	+3·34	−12·1	+0·78
	γ-Hydroxybutyrate	−19·3	+3·59	−11·9	+1·27
Rat					
5	None	—	—	− 7·1	−0·26
	γ-Hydroxybutyrate	−27·4	+6·9	− 7·2	−0·76
	dl-αγ-Dihydroxy-butyrate	−26·7	+5·7	− 8·5	+0·17
6	None	−18·8	+1·97	− 8·4	−2·31
	Crotonate	−21·3	+2·31	− 9·6	−1·53
	trans-γ-Hydroxy-crotonate	−24·6	+9·05	− 9·4	+1·97

Table 9. *Formation of β-ketonic acids from 4-carbon monocarboxylic acids in liver tissue*

(3 ml. phosphate saline; tissue; 0·0156 M substrate; O_2; 60 min.)

Exp. no.	Substrate added	Q_{O_2}	$Q_{\beta\text{-ket. acid}}$
Pigeon liver			
1	None	−10·5	+0·55
	Butyrate	−11·1	+2·00
	Crotonate	−11·8	+2·2
	α-Hydroxybutyrate	−13·1	0·00
	β-Hydroxybutyrate	−12·3	+2·4
	γ-Hydroxybutyrate	−15·2	+0·95
	trans-γ-Hydroxycrotonate	−11·8	+0·65
	dl-βγ-Dihydroxybutyrate	−13·0	+1·22
	Tetrolate	−11·0	+0·85
	Tetronate	−11·1	+0·61
	Vinylglycollate	− 7·7	0·00
Rat liver			
2	None	−10·7	+0·82
	Butyrate	−12·6	+2·87
	Crotonate	−14·5	+5·58
	Vinylacetate	−14·6	+6·52
	dl-αγ-Dihydroxybutyrate	−13·5	+0·90
	dl-βγ-Dihydroxybutyrate	−13·4	+2·16
	γ-Hydroxybutyrate	−14·4	+0·82
3	None	−10·8	+0·75
	Butyrate	−12·8	+2·47
	Crotonate	−13·2	+4·43
	Vinylacetate	−13·6	+5·00
	β-Hydroxybutyrate	−13·1	+6·15

Formation of β-ketonic acids from butyric and related acids in liver. It is known that acetoacetic acid is formed in the liver from butyric, β-hydroxybutyric [Embden, Salomon & Schmidt, 1906], and crotonic [Friedmann, 1908] acids. Lipmann & Perlmann [1942] have shown that tetrolic acid does not give rise to acetoacetic acid in rat liver. A study was made of the formation, in the liver tissue of starving pigeons and rats, of β-ketonic acids from certain 4-carbon monocarboxylic acids not hitherto examined (see Table 9). β-Ketonic acids are formed from butyric, crotonic, β-hydroxybutyric, vinylacetic, and, to a small extent, from *dl*-βγ-dihydroxybutyric acids. None of the other substrates tested was appreciably ketogenic.

In the rat liver vinylacetic acid readily gives rise to acetoacetic acid. $Q_{acetoac.}$ exceeded, in our experiments, that from butyric and crotonic acids, but was lower than that from β-hydroxybutyric acid.

No β-ketonic acid is formed from vinylacetic or *dl*-βγ-dihydroxybutyric acid under anaerobic conditions (Table 10).

Table 10. *Formation of β-ketonic acids in starved rat liver under aerobic and anaerobic conditions*

Substrate (0·0156 M)	O_2 Q_{O_2}	O_2 $Q_{\beta\text{-ket. acid}}$	N_2 $Q_{\beta\text{-ket. acid}}$
0	− 9·95	+0·86	0·00
Crotonate	−11·5	+3·08	0·42
Vinylacetate	−14·0	+5·85	0·15
dl-βγ-Dihydroxy-butyrate	−12·5	+2·53	0·36

DISCUSSION

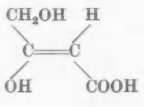
Blixenkrone-Møller [1938] found that β-hydroxybutyric acid does not give rise to carbohydrate in the perfused liver of cats, whilst butyrate does, and concluded that β-hydroxybutyric acid is not an intermediate in the formation of carbohydrate from butyric acid. He therefore suggested that butyric acid is oxidized in animal tissue both at the β-carbon atom, yielding ketone bodies, and at the γ-carbon atom, yielding succinic acid, which is then further oxidized.

The results of our experiments suggest that β-hydroxybutyric acid is not an intermediate on the main pathway of the oxidation of butyric acid in rat kidney. It would appear therefore that the formation of ketone bodies represents an alternative path in the oxidation of butyric acid in the animal. It is interesting to note, in view of Blixenkrone-Møller's suggestion of γ-oxidation of butyric acid, that γ-hydroxybutyric acid is oxidized at a higher rate than butyric acid in both guinea-pig and rat kidney slices.

Under our experimental conditions, vinylacetic acid is oxidized in kidney slices at a higher rate than butyric acid. With vinylacetic acid a higher rate of β-ketonic acid formation than with butyric acid as substrate is also observed in starved rat liver. The compound thus shows physiological properties similar to those of butyric acid, and it seems possible that vinylacetic acid is an intermediate in the breakdown of butyric acid.

In our experiments a β-ketonic acid is formed in liver tissue from starving animals, in the presence of *dl*-$\beta\gamma$-dihydroxybutyric acid. Tetronic acid, which would be expected to arise from the oxidation of the β-hydroxyl of *dl*-$\beta\gamma$-dihydroxybutyric acid, does not behave as a β-ketonic acid (it does not yield any CO_2 on treatment with aniline citrate: personal communication from Dr H. A. Krebs) and consequently it cannot be the β-ketonic acid formed in the presence of *dl*-$\beta\gamma$-dihydroxybutyric acid.

The β-ketonic acid might be acetoacetic acid formed from substrates present in the liver tissue; such an effect would be similar to that observed when malonic acid is added to liver tissue [Jowett & Quastel, 1935]. *dl*-$\beta\gamma$-Dihydroxybutyric acid, however, increases Q_{O_2}, whilst malonate inhibits it. The possibility of the formation of acetoacetic acid from this substrate by dehydration seems unlikely in view of the observation that no β-ketonic acid is formed under anaerobic conditions from this substrate. On the other hand, it seems possible that a structural isomer of tetronic acid, hitherto unknown, may be formed, viz. the *trans*-isomer (I) of the enolic form of tetronic acid as opposed to the *cis*-isomer (lactone form, II):



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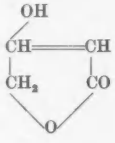

II

SUMMARY

1. The oxidation, in kidney slices of well-fed guinea-pigs and rats, of the Na-salts of butyric, crotonic, vinylacetic, tetrolic, α-hydroxybutyric, β-hydroxybutyric, γ-hydroxybutyric, *trans*-γ-hydroxycrotonic, *dl*-$\alpha\gamma$-dihydroxybutyric, *dl*-$\beta\gamma$-dihydroxybutyric, tetronic, and vinylglycollic acids was studied by comparing the Q_{O_2} and the formation of bicarbonate in the presence of these substrates with that of a control experiment without substrate. Of the above substrates butyric, crotonic, vinylacetic, β-hydroxybutyric, γ-hydroxybutyric, *dl*-$\alpha\gamma$-dihydroxybutyric acids are rapidly oxidized in the kidney of both species. *trans*-γ-Hydroxycrotonic acid is oxidized in rat kidney only. Vinylglycollic acid inhibits tissue respiration. α-Hydroxybutyric, tetrolic, *dl*-$\beta\gamma$-dihydroxybutyric and tetronic acids are not appreciably oxidized.

2. From rates of bicarbonate formation in the presence of butyric, crotonic, vinylacetic, and β-hydroxybutyric acids in rat kidney slices, it is concluded that β-hydroxybutyric and crotonic acids are oxidized more slowly than is butyric acid. In the presence of vinylacetic acid $Q_{bic.}$ is higher than it is in the presence of butyric acid.

3. In rat kidney slices formation of an unsaturated compound in the presence of butyric and β-hydroxybutyric acids occurs under aerobic conditions.

4. Under anaerobic conditions vinylacetic acid disappears in rat kidney slices.

5. 0·031 M malonate inhibits, in the kidney of both species, the oxidation of all the substrates tested.

6. Vinylacetic acid and *dl*-$\beta\gamma$-dihydroxybutyric acid are ketogenic in liver slices from starved pigeons and rats.

7. It is suggested that vinylacetic acid may play a part in the intermediate metabolism of butyric acid in animal tissue.

I am very grateful to Dr H. A. Krebs for his constant advice and criticism during this investigation.

REFERENCES

Blixenkrone-Møller, N. [1938]. *Hoppe-Seyl. Z.* **252**, 117; 137.
Cross, M. C. A. & Holmes, E. [1937]. *Brit. J. exp. Path.* **18**, 370.
Dakin, H. D. [1922]. *Oxidations and Reductions in the Animal Body*, 2nd ed. London: Longmans, Green.
Edson, N. L. [1935]. *Biochem. J.* **29**, 2082.
Embden, G., Salomon, H. & Schmidt, Fr. [1906]. *Beitr. chem. Physiol. Path.* **8**, 129.
Feist, F. [1906]. *Liebigs Ann.* **345**, 104.
Fichter, Fr. & Herbrand, A. [1896]. *Ber. dtsch. chem. Ges.* **29**, 1192.
Friedmann, E. [1908]. *Beitr. chem. Physiol. Path.* **11**, 371.
Hoff-Jørgensen, E. [1940]. *Hoppe-Seyl. Z.* **266**, 56.
Johnson, W. A. [1939]. *Biochem. J.* **33**, 1046.
Jowett, M. & Quastel, J. H. [1935]. *Biochem. J.* **29**, 2143, 2159, 2181.
Kleinzeller, A. [1943]. *Biochem. J.* **37**, 674.
Linstead, R. P., Noble, E. G. & Boorman, E. J. [1933]. *J. chem. Soc.* p. 557.
Lipmann, F. & Perlmann, G. E. [1942]. *Arch. Biochem.* **1**, 41.
Meyerhof, O. & Lohmann, K. [1926]. *Biochem. Z.* **171**, 381.
Nef, J. U. [1910]. *Liebigs Ann.* **376**, 1.
—— & Dar Juan, T. [1910]. *Liebigs Ann.* **376**, 35.
Quastel, J. H. & Wheatley, A. H. M. [1935]. *Biochem. J.* **29**, 2773.
Rambaud, R. [1935]. *Bull. Soc. chim. Fr.* (5), **1**, 1317.
van der Sleen, M. G. [1902]. *Rec. Trav. chim. Pays-Bas*, **21**, 209.
Weil-Malherbe, H. [1938]. *Biochem. J.* **32**, 1033.
Wolff, L. & Schwabe, C. [1896]. *Liebigs Ann.* **291**, 231.

Spectrographic Determination of Lead in Blood

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Although application of spectrographic methods of analysis to the study of the physiology of the 'trace elements' has been made in this country by Sheldon & Ramage [1931] and by Kent & McCance [1941], little study has been devoted to trace elements from the aspect of industrial metallic poisoning. In America, Cholak [1935] described a spectrographic method for the analysis of lead in blood, using bismuth as the internal reference element. This paper describes a spectrographic method adopted in the investigation of suspected industrial lead poisoning, and some of the results obtained.

METHODS

General. All analytical material was collected and prepared for spectrographing with strict precautions to prevent contamination, by ensuring that, where possible, the vessels and reagents used were Pb-free. Loss of analytical material was eliminated by reducing the handling and chemical treatment of the samples to a minimum.

Spectrograph. The large Hilger quartz spectrograph, which has a range of 1910–8000 A., was used with the standard conditions outlined in Table 1. For the Pb investigation the region 2700–4300 A. was employed. Identification of the lines was achieved by use of the Judd Lewis Comparator, an instrument which permits of simultaneous microscopic examination of the test plate and of a standard plate showing the position of all the elements. The line densities of emission spectra are in direct ratio to molecular concentration in a sample of their elements of origin. By use of the Hilger non-recording microphotometer to measure the ratio of the line densities of different known quantities of Pb, and a constant amount of an internal reference element, a graph can be prepared which provides a means of measurement of the unknown amount of Pb in a sample.

Table 1. *Standard conditions for spectrograph*

Wave band	2700–4300 A.
Slit width	0·015 mm. (fixed slit)
Arc gap	2 mm.
Voltage	120 d.c.
Amperage	5
Upper electrode	H.S. graphite pointed to 80° angle
Lower electrode	H.S. graphite 10 mm. diam. cut to form a crater to receive sample
Exposure	1 min.
Plate	Ilford H. and D. 70
Development:	(*a*) 90 sec. at 19·5°
	(*b*) Fixed, acid hypo 15 min.
	(*c*) Washed running water 15 min.
	(*d*) Dried in current of warm air

Standard of internal reference. In our earlier investigations the bismuth line at 2898 A. was chosen as the internal reference line for measurement against the Pb line at 2833 A., with satisfactory accuracy when checked by the addition of known quantities of Pb to duplicate samples of blood and synthetic ash. Investigation of subjects exposed to Bi of therapeutic origin confirmed our suspicions that Bi could be encountered normally in blood, and invalidated its use as a standard (Pl. 1, fig. 1).

After investigation of several elements, it was decided to use platinum as the internal reference element, since it fulfilled all the conditions required, and its line at 2830 A. is in a position of such proximity to that of Pb at 2833 A. as is ideal for measurement (Fig. 1). After experiment it was found that the most satisfactory Pt solution to add to the ash specimens, following solution in aqua regia, was one containing 0·01 g./100 ml. H_2SO_4. We consider it worth while to draw attention to the use of Pt in the analysis of several other elements implicated in poisoning by heavy metals. By varying the amount of Pt used, and if necessary the exposure time and type of plate in accordance with the spectrographic sensitivity of the element under investigation, Pt could be used as the internal reference element for the following metals:

As	2860·5 A.	Ni	3002 A.	Cr	2986·4 A.	Au	2676 A.
Pt	2830 A.	Pt	2998 A.		3015 A.	Pt	2702 A.
				Pt	2988 A.		
Th	2837 A.	V	3063·7 A.	Mn	2798·3 A.	Cd	2980 A.
Pt	2830 A.	Pt	3064·7 A.	Pt	2830 A.	Pt	2998 A.

With few exceptions all the above pairs of lines are those of *raies ultimes*. All the Pt lines are of high spectrographic intensity and are not overshadowed, which facilitates accurate measurement of line densities. Where the internal reference line is overshadowed by close juxtaposition of another element line, it may be difficult to be certain that the overshadowing line is not in part responsible for the deflexion on the galvanometer scale which is attributed to the internal reference line. Kent & McCance must have experienced this difficulty in their analysis of Au (2676·6 A.), with Mg (2795·5 A.) as the internal reference line. Consultation of Harrison's wave-length tables indicates a line of Fe at 2795·54 A., i.e. 0·01 A. difference.

Standard ash. The standard material used for preparation of the graph was a synthetic ash which gave a main base spectrum similar to that of blood. Persistent traces of Pb were found in all the supposedly pure chemical reagents used, even after prolonged refining by chemical and electrolytic methods. Spectrographic examination showed that the ash finally contained 1–2 μg. Pb/100 g.

To a number of samples of synthetic ash (0·05 g. the approximate weight of the ash of the blood samples used) different known amounts of Pb and a constant amount of Pt were added. The samples (duplicate) were then spectrographed. The curve from the mean of two sets of results was taken as the standard graph (Fig. 2). It is advisable to use the logarithmic ratios of the line densities, as smooth

working curves are obtained (Fig. 2). Some of the results from a series of identical samples used to check the accuracy of the graph are shown in Table 2. Errors of more than $\pm 8\mu g.$/100 ml. of blood are exceptional, provided that the standard spectrographic conditions are strictly adhered to.

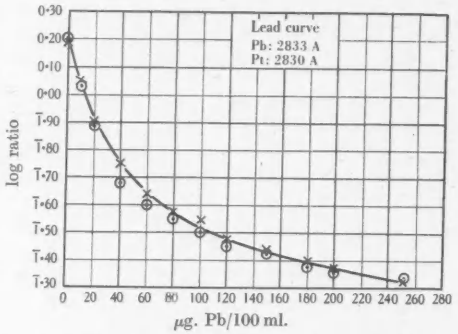

Fig. 2. Lead curve. Relation of spectrographic line intensity ratios to quantity of Pb. Ordinates, log intensity Pb 2833 A./Pt 2830 A.; abscissae, $\mu g.$ Pb.

Table 2. *Recovery of added lead*

Material analyzed	Ash weight (g.)	Pb added ($\mu g.$/100 ml.)	Pb found ($\mu g.$/100 ml.)	Error ($\mu g.$/100 ml.)
A. 5 ml. whole blood	0·0506	—	18	—
5 ml. whole blood and Pb	0·0522	40	60	+2
B. Whole blood	0·0516	—	17	—
Whole blood and Pb	0·0526	80	100	+3
Standard ash 1 + Pb	0·0500	50	48	−2
Standard ash 2 + Pb	0·0500	100	108	+8
Standard ash 3 + Pb	0·0500	120	116	−4
Standard ash 4 + Pb	0·0500	140	138	−2

Treatment of samples. 5 ml. of blood, urine, etc., were collected, transferred to a Pb-free silica crucible and sulphated by the addition of 3 ml. Pb-free H_2SO_4. The samples were then ashed in a dust-proof electric furnace by treatment at 110° for 12 hr., followed by 500° for 8 hr.

The low Fe content of serum, urine and cerebrospinal fluid gives spectrographically what is known as a weak base spectrum, which would be a source of error in applying the blood Pb curve to their analysis, as was pointed out by Foster, Langstroth & McCrae [1935]. We therefore added 0·02 g. of synthetic ash to samples of serum and urine; in this way their main base spectrum was intensified to resemble that of the standard samples. Experiment showed that the results obtained with serum and urine were accurate to $\pm 10\mu g.$/100 ml. As yet we have not obtained sufficient quantities of cerebro-spinal fluid to check the application of the Pb curve; we assume that since its main base spectrum is similar to that of serum and urine, the error, when the synthetic ash additions are made, will be of the same magnitude. By this method it is possible to report results on 20 samples within 24 hr. after collection, with minimal use of chemical reagents and vessels.

RESULTS

In a series of 80 normal unexposed subjects we have found the mean concentration of Pb in the blood to be $40\mu g.$; the results ranged from 5 to $120\mu g.$/100 ml. of blood, and the majority fell between 20 and $60\mu g.$

As was expected, those individuals who were exposed to the swallowing of metallic dusts, or inhalation of metal fumes, showed higher concentrations than office workers and the like. In four office workers, for example, the mean concentration was $16\mu g.$, compared with $88\mu g.$ in four metal workers. These latter findings are similar to those of Kehoe, Thamman & Cholak [1933*a*] and of Cholak & Bambach [1943].

The maximum normal concentration is the only thing that matters, since the concentration in an individual varies widely within normal limits from week to week. We have found, in sampling the same three individuals at intervals of four weeks, variations from $21–73\mu g.$, $150–53\mu g.$ and $43–11\mu g.$ respectively. One of us (A.T.) has found a similar variation in blood Pb concentration throughout the day. Although occupational exposure is mainly responsible for this variation in individual concentrations, the Pb content of the diet also contributes to it. From an analysis of foodstuffs we have found bread to contain $168\mu g.$ of Pb/100 g., tea biscuit $216\mu g.$/100 g., sausage and cheese 85 and $43\mu g.$/100 g. Some of the Pb content of these foodstuffs originates no doubt in contamination from vessels. That the Pb content of the diet contributes materially to blood Pb concentrations, conflicts with the view that there is little or no alimentary absorption of Pb by reason of the insoluble nature of the Pb compounds formed during the process of digestion. The total Pb in the circulation is, however, small in amount, and absorption of very small quantities may suffice to cause what is physiologically a substantial increase in its concentration in the blood. The following results (Table 3) show the effects on blood and urine concentrations of 12·5 grains lead

Table 3. *Effect of ingesting lead on the content of blood and urine*

	Subject	$\mu g.$ Pb/100 ml. before ingestion	$\mu g.$ Pb/100 ml. after ingestion
Blood	E.	11	250
	T.	14	155
	McE.	21	49
	B.	23	58
Urine	E.	5	107
	T.	3	72
	McE.	5	45
	B.	5	26

(5 ml. samples of blood or urine were analyzed.)

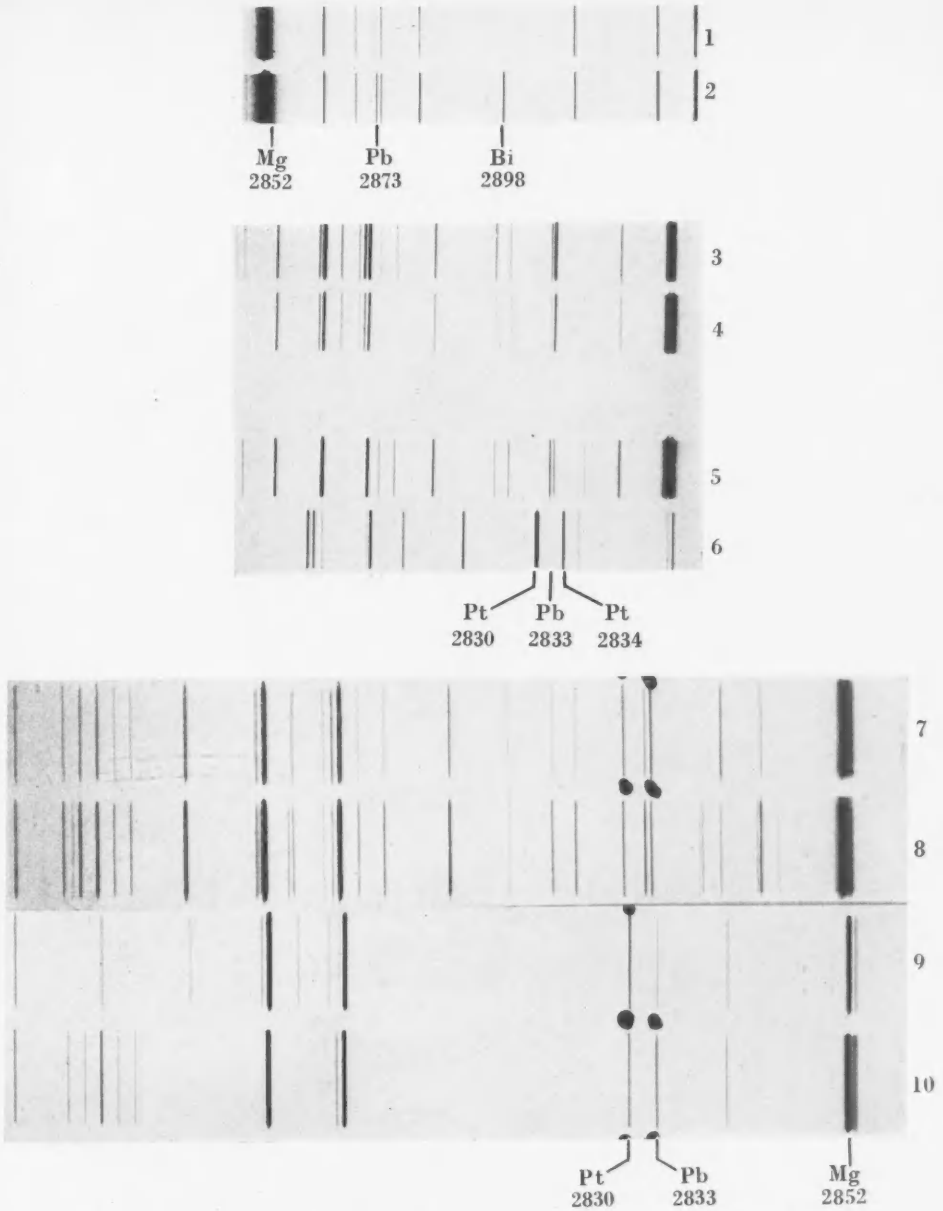

Fig. 1. Blood spectrograms. 1, shows Bi absent, while 2 denotes its presence due to absorption. 3, 4 and 5, blood spectrograms compared with a pure Pt spectrum 6 (note proximity of Pb 2833 A. to Pt 2830 A. and Pt 2834 A.). Variation in blood Pb content is evident from densities of Pb 2833 A. in 3, 4 and 5. 7 and 8, mother and infant bloods (case E). 9 and 10, infant urine and milk collected 5th day (case E); presence of Pb throughout clearly indicated, as also reduction of Fe lines in milk and urine.

acetate swallowed in the form of pills over three days by four subjects, who experienced no ill effects therefrom.

The bladder was emptied ½ hr. before and the urine for test collected ½ hr. after the blood was drawn. This gave an indication of the rate of excretion for a given blood concentration. From these results it is clear that considerable elevation of the blood Pb is possible by absorption of small quantities of Pb from the intestine.

Investigations on the blood of newly born infants shows that the Pb concentration approximates to that of the maternal blood. The investigation was carried out as follows. In the second stage of labour just before delivery 5 ml. of blood were withdrawn from the mother's arm, and 5 ml. from the umbilical vein prior to severance of the cord. The samples were covered and treated as described for spectrographing (A, B, C, Table 4, Fig. 1). In another similar experiment, samples of the maternal milk and urine from the infant on the fifth day (D, E, F, G, Table 4, Fig. 1) were analyzed.

Table 4. *Lead content of maternal and of infant blood, urine and milk*

	Lead (μg. Pb/100 ml.) in			
	Maternal		Infant	
Subject	Blood	Milk	Blood	Urine
A	54	—	57	—
B	43	—	42	—
C	34	—	28	—
D	46	32	71*	2
E	22	8	37	7
F	33	20	18	3
G	35	5	25	—

* Doubtful result: contamination probable.

DISCUSSION

Some diversity of results has been reported for normal blood Pb concentrations by different workers. Blumberg & Scott [1935], by visual comparison of the line densities, found the normal to vary from 5 to 100 μg./100 ml.; a critical concentration between 100 and 150 μg. produced symptoms. Kehoe, Thamman & Cholak [1935], using Bi 2898 A. as the internal reference element, reported 58 μg./100 ml. to be the mean concentration in a series of 71 cases; this to us appears high. We agree with Blumberg & Scott that the maximum normal concentration is 100 μg./100 ml. and consider that, in cases of chronic exposure, the critical concentration is around 130 μg./100 ml. of blood. Although we agree with Tompsett & Anderson [1939], that the blood and urine Pb do not run parallel, it is evident that, in health, elevated blood concentrations are balanced by rapid excretion of the Pb absorbed. Our results further indicate that some control over diet and occupational exposure is necessary in the investigation of suspected Pb poisoning, and that errors in diagnosis may arise from single analyses. The Pb content of the diet, and occupational exposure, are, no doubt, accountable for some of the divergencies in results reported by different observers. One other possible factor in the variation of results is the Pb transmitted from mother to foetus. The amount is surprising, as also are the facts that the infant appears to retain it, and that the Pb content of the milk is sufficient to maintain the infant's original concentration. The results on human milk are higher than those reported by Kehoe *et al.* [1933*b*]. This may possibly be attributable to differences in time of sampling and sensitivity of the analytical method used. The unfailing appearance of Pb in blood and tissue fluids, and its transmission to the foetus, raises the question as to whether Pb has a physiological function as yet undiscovered. The opportunities for such investigations are few and collection of the necessary samples considerably more difficult than might be thought. In spite of the use of Pb-free crucibles and test-tubes for direct collection of milk and infant urine, occasional minute contamination is inevitable.

SUMMARY

1. A spectrographic method of lead analysis in blood is described, using platinum as the internal reference element.

2. Blood lead concentrations encountered in 80 healthy subjects ranged from 5 to 120 μg./100 ml. with a mean of 40 μg./100 ml. and greatest frequency of from 40 to 60 μg./100 ml. More than 50 % of the results lie between 20 and 60 μg.

3. Lead is transmitted to the foetus in amounts approximating to those of the maternal circulation.

4. Lead is present in human milk and is very slowly excreted in infant urine.

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REFERENCES

Blumberg, H. & Scott, T. F. McN. [1935]. *Johns Hopk. Hosp. Bull.* **56**, 276.
Cholak, J. [1935]. *Industr. Engng Chem.* (Anal. ed.), **7**, 287.
—— & Bambach, K. [1943]. *J. industr. Hyg.* **25**, 47.
Foster, J., Langstroth, G. O. & McCrae, D. R. [1935]. *Proc. roy. Soc.* A, **153**, 141.
Kehoe, R. A., Thamman, F. & Cholak, J. [1933*a*]. *J. industr. Hyg.* **15**, 306.
—— —— —— [1933*b*]. *J. industr. Hyg.* **15**, 302.
—— —— —— [1935]. *J. Amer. med. Ass.* **104**, 90.
Kent, N. L. & McCance, R. A. [1941]. *Biochem. J.* **35**, 837.
Sheldon, J. H. & Ramage, H. [1931]. *Biochem. J.* **25**, 1608.
Tompsett, S. L. & Anderson, A. B. [1939]. *Lancet*, **1**, 553.

The Vitamin B-complex Requirements of Several Insects

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It has been shown by us [Fraenkel & Blewett, 1943*b*] that six insects, which normally feed on flour and other dried food, grow satisfactorily on a diet of casein 50, glucose 50, yeast 5, cholesterol 1, McCollum's salt mixture 1, and water 15 parts, but entirely fail to grow when yeast is omitted. With the yeast replaced by yeast extract, the growth rate is considerably slower. No growth takes place if the diet contains a water-insoluble fraction alone in place of whole yeast. Previous work has indicated that insects require several vitamins of the B-group; the position up to 1940 has been reviewed by Craig & Hoskins [1940] and Trager [1941]. The present paper deals with the growth factors required by insects which are contained in the water-soluble and in the water-insoluble fractions of yeast. Short accounts of some of the results have already appeared elsewhere [Fraenkel & Blewett, 1942 *a*, *b*; 1943*a*, *d*].

EXPERIMENTAL

The following beetles were used: *Tribolium confusum* Duv., Tenebrionidae; *Silvanus* (*Orycaephilus*) *surinamensis* L., Cucujidae; *Sitodrepa panicea* L., Anobiidae; *Lasioderma serricorne* Fab., Anobiidae; *Ptinus tectus* Boield., Ptinidae.

The following preparations and substances were used to compound diets:

Casein A/E, vitamin and fat free (Glaxo Laboratories Ltd.). Dried debittered brewer's yeast (Glaxo Laboratories Ltd.). Yeast extract, prepared from fresh brewer's yeast according to Chick & Roscoe [1930]. 'Insoluble yeast': residue from preparation of yeast extract, after 10 successive extractions with large volumes of boiling water.

Aneurin hydrochloride (B_1) (Glaxo Laboratories Ltd.), riboflavin (Glaxo Laboratories Ltd.), nicotinic acid (British Drug Houses Ltd.), pyridoxin (Glaxo Laboratories Ltd.), Capantothenate (Glaxo Laboratories Ltd. and Roche Products Ltd.), choline chloride (British Drug Houses Ltd.), *p*-aminobenzoic acid (British Drug Houses Ltd.), *i*-inositol (British Drug Houses Ltd.), biotin methyl ester (Research Laboratories, S.M.A. Corporation), and biotin (Prof. du Vigneaud).

All the tests were performed in 2 × 1 in. specimen tubes. Each tube contained 2 g. of the dry diet and each diet was prepared in duplicate. Each test was performed with 20 larvae, 10 in each tube. The tubes were kept in a room of which the temperature was automatically maintained at 25° and the relative humidity at 70%. The criteria for assessing results are rate of growth from the newly hatched larva until pupation (*Tribolium*) or emergence of adult (other insects), and mortality. The methods of experimenting and assessing results are fully described elsewhere [Fraenkel & Blewett, 1943 *b*, *c*].

Preparation of a diet deficient in riboflavin. Yeast extract was treated with norit charcoal (Hopkin and Williams, Ltd.) according to the method of El Sadr, Macrae & Work [1940]. The yeast extract was not previously autoclaved, but as a precaution aneurin HCl was added to the diet, which then consisted of casein 50, glucose 50, cholesterol 1, yeast charcoal filtrate 15, insoluble yeast 5, McCollum's salt mixture 1 parts, aneurin HCl 50 μg./g. Riboflavin was added to the diet in graded doses and the results showing a graded response (Table 1)

Table 1. *Pupation of* Tribolium *on a diet deficient in riboflavin, with the addition of graded doses of riboflavin*

Riboflavin (μg./g. of diet)	No. of pupae	Spread in the pupation period (days)
None	11	60–113
0·625	16	50– 74
1·25	18	32– 43
2·5	16	29– 39
5·0	18	30– 39
10·0	16	30– 39
50·0	20	28– 39
Wholemeal flour (control)	19	27– 30

confirmed those reported by Fröbrich [1939], who grew *Tribolium* on a diet containing yeast extract which had been heated in an alkaline medium in order to destroy the riboflavin.

Substitution of yeast extract by pure vitamins. As pure vitamins of the B-group became available, attempts were made to feed mixtures of pure vitamins instead of yeast extract. Except in some earlier tests, wheat-germ oil was added to the diets

as a precaution against possible deficiencies of unknown fat-soluble factors. The vitamins were dissolved separately in a small quantity of water and then mixed so that 0·1 ml. of the mixture contained the precise amount required for each tube. The diets used in subsequent tests are recorded in Table 2.

Table 2. *Diets used in the later series of experiments*

		No. and composition of diet used in corresponding series of experiments					
		1	2	3	4	5	6
Casein (parts)		50	50	50	50	50	50
Glucose (parts)		50	50	50	50	50	50
Cholesterol (parts)		1	1	1	1	1	1
'Insoluble yeast' (parts)		5	5	5	5	5	5
McCollum's salt mixture no. 185 (parts)*		1	1	1	1	1 or 3	2
Wheat germ oil (mg./g.)		—	—	15	15	15	15
Water (parts)		15	15	15	15	15	15
Aneurin HCl	μg./g.	50	50	50	50	50	50
Riboflavin	μg./g.	50	50	50	50	50	50
Nicotinic acid	μg./g.	50	50	50	50	50	50
Pyridoxin	μg./g.	50	50	50	50	50	50
Ca-pantothenate	μg./g.	—	50	50	50	50	50
Choline chloride	μg./g.	50	50	50	50	500	500
p-Aminobenzoic acid	μg./g.	—	—	50	50	50	50
Inositol	μg./g.	—	—	—	50	500	500

* A modification of McCollum's mixture no. 185. Formula in Gay [1938].

RESULTS

Growth requirements of Tribolium confusum

The results with *Tribolium confusum* on different diets are recorded in Fig. 1. It is seen that addition

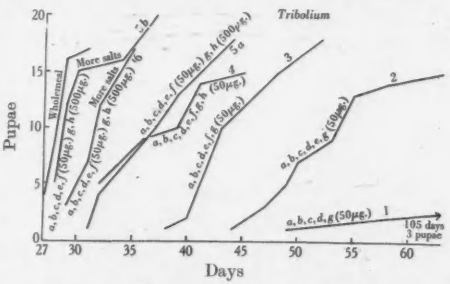
Tribolium
Pupae
Days
Wholemeal
More salts
a, b, c, d, e, f (50μg.) g, h (500μg.)
a, b, c, d, e, f (50μg.) g, h
a, b, c, d, e, f, g, h (50μg.)
a, b, c, d, e, f, g (50μg.)
a, b, c, d, e, g (50μg.)
a, b, c, d, g (50μg.)
105 days 3 pupae

Fig. 1. Growth of *Tribolium confusum* on a diet of casein, glucose, cholesterol, 'insoluble yeast', salts and water, to which vitamins of the B-complex have been added in various combinations. Numbers on the curves refer to number of diet in Table 2. *a* = aneurin, *b* = riboflavin, *c* = nicotinic acid, *d* = pyridoxin, *e* = pantothenic acid, *f* = *p*-aminobenzoic acid, *g* = choline chloride, *h* = inositol. Quantities of vitamins given/g. of the dry diet.

of pantothenic acid in series 2 greatly improved the diet. In the third series *p*-aminobenzoic acid and wheat-germ oil, and in the fourth series inositol were added, again noticeably improving the diet. In view of the large amounts of inositol and choline required by vertebrates, these substances were given in amounts 10 times as large as those of the other vitamins in the fifth series. The full diet (Fig. 1, curve 5*a*) was now much better than the corresponding one of series 3 (curve 3), but still inferior to the control. With the quantities of each vitamin halved, the diet was not affected, probably indicating that the quantities were sufficient and also not large enough to cause toxic symptoms. Addition of more 'insoluble yeast' (15 % instead of 5 %) improved the diet somewhat, but a greater improvement was achieved by adding 1 % whole yeast. It is unlikely that this improvement was due to the supply of additional undetermined essential B-factors, because the same kind of improvement was obtained by approximately trebling the amount of salt mixture (cf. curve 5 *a* and *b*, Fig. 1). The diet now became virtually as effective as wholemeal flour. It therefore appeared that any missing factor supplied by 1 % yeast was a mineral.

A sixth diet was devised for *Tribolium* which contained twice as much salts as previously and it was compared with diets in which one of the vitamins was omitted at a time (Fig. 1, curve 6; Fig. 2). This diet proved of the same value as a diet which contained aqueous yeast extract in place of pure vitamins. In this series, but not in series 5, the diet was slightly inferior to wholemeal flour, but this difference is probably not significant. Without riboflavin, nicotinic acid, or pantothenic acid the diet

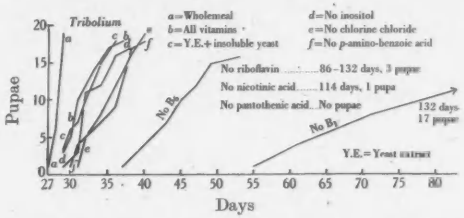

Fig. 2. *Tribolium confusum*. Growth on diet 6 (Table 2), compared with similar diets from which single vitamins have been left out one at a time.

became ineffective; without pyridoxin growth became slower, and still more so without aneurin. Omission of *p*-aminobenzoic acid or choline chloride produced a noticeable delaying effect, while absence of inositol impaired the efficiency of the diet only very slightly (Fig. 2).

All these diets contained a possible source of vitamins in the 'insoluble yeast' fraction which might have retained significant amounts of some factors even after prolonged extraction. Meanwhile the missing factor in 'insoluble yeast' had been found to be biotin (p. 689). It then became possible to perform, simultaneously with the tests of series 6, a parallel series in which 'insoluble yeast' was re-

placed by biotin (0·1 μg./g.) [Fraenkel & Blewett, 1943*a*, Fig. 3]. The results of these two series, one containing 'insoluble yeast' and the other biotin, were virtually identical, with three exceptions. In the biotin series absence of inositol delayed growth significantly, whereas absence of *p*-aminobenzoic acid had no effect. A more serious discrepancy applied to diets without pantothenic acid. These were totally inefficient in the 'insoluble yeast' series, but not quite so deficient in the biotin series, where 13 out of 20 larvae completed development, although at a greatly reduced rate. This discrepancy cannot at present be explained. It appears from a comparison of these two sets of tests that no significant amounts of vitamins could have been added to diets with 5% 'insoluble yeast'. Inositol is the only factor where the slight discrepancy in the results may, possibly, be due to retention of inositol in 'insoluble yeast'. An aneurin determination on our 'insoluble yeast' made by Dr Y. L. Wang (Cambridge) showed below 0·1 μg./g., a quantity too small to have any effect.

Growth requirements of other insects

Concurrently with the tests of diet 6 performed with *Tribolium*, the diets which contained 'insoluble yeast' were also tested with four other beetles. The results are summarized in Table 3.

The complete diet was as good, if not better, than the controls which contained yeast extract as a source of B-vitamins. Omission of inositol or *p*-aminobenzoic acid produced a slight, but possibly significant delay in growth. Absence of choline had a very pronounced delaying effect (Fig. 3), much greater than with *Tribolium*. Absence of the other vitamins had an effect similar to that described for *Tribolium*.

Lasioderma serricorne gave results quite different from *Tribolium* and *Ptinus*. Absence of riboflavin, nicotinic acid, *p*-aminobenzoic acid or inositol seemed to have no effect. Absence of aneurin, pyridoxin or pantothenic acid delayed development considerably, but to a much slighter extent than was the case with *Tribolium* and *Ptinus*. Absence of choline chloride had a very pronounced delaying action (Table 3, and Fraenkel & Blewett [1943*a*, Fig. 2]).

Sitodrepa panicea also gave results different from *Tribolium* and *Ptinus*, and from *Lasioderma*. The growth rate on the complete diet was considerably inferior to that of the control, where yeast extract was given instead of pure B-vitamins. Absence of nicotinic acid, inositol, choline chloride or *p*-aminobenzoic acid greatly affected the efficiency of the diet, which became totally inefficient only in the absence of aneurin (Table 3, and Fraenkel & Blewett [1944, Fig. 4]).

Table 3. *The effect on the growth of various Coleoptera larvae of the omission from the diet (Table 2, no. 6) of different members of the B-group of vitamins*

Description of diet	*Tribolium*			*Ptinus*			*Silvanus*			*Lasioderma*			*Sitodrepa*		
	Growth rate	Days	Nos.	Growth rate	Days	Nos.	Growth rate	Days	Nos.	Growth rate	Days	Nos.	Growth rate	Days	Nos.
Wholemeal flour	114	27– 29	19	85	57–67	16	115	25–29	15	94	35–39	20	97	37–41	17
Yeast extract + 'insoluble yeast'	100	29– 36	18	100	47–58	18	100	27–35	16	100	34–39	19	100	37–39	15
All vitamins (Table 2, no. 6)	100	29– 37	18	104	47–60	18	94	27–36	16	92	34–41	19	90	38–45	13
No aneurin	42	55–132	17	70	70–79	2	97	27–36	16	81	41–47	18	45	74–94	4
No riboflavin	31	86–132	3	0	—	0	94	30–46	9	97	34–41	18	88	41–49	9
No nicotinic acid	27	114	1	66	79	1	78	51	1	97	34–39	18	93	40–47	12
No pyridoxin	70	37– 53	16	63	69–90	13	91	31–41	16	87	36–48	17	84	43–47	14
No pantothenic acid	0	—	0	70	69–77	2	103(?)	30	1	87	36–41	8	93	39–49	5
No choline chloride	88	31– 40	19	74	64–83	14	89	32–36	16	74	43–61	14	90	38–51	12
No inositol	97	29– 38	17	98	48–58	17	91	29–37	15	97	34–41	18	90	41–45	13
No *p*-aminobenzoic acid	86	30– 40	18	98	52–60	18	94	30–41	14	97	34–41	18	90	39–47	14

The growth rate (col. 1, under appropriate insect heading) is calculated as a reciprocal of the period in which half the number of insects completed their development and is expressed as a percentage of the controls. The period of development (col. 2) records the spread in the larval period (*Tribolium*) or larval plus pupal period (other insects). Col. 3 records the number, out of 20, completing development. The 'all vitamins' diet was that recorded in Table 2, no. 6. The relative growth rates of *Tribolium* and the other insects are not strictly comparable because they are based with *Tribolium* on the larval period only and with the other insects on the larval plus pupal period. Length of the pupal period of the insects in question is approximately 10 days.

Ptinus tectus [Fraenkel & Blewett, 1943*a*, Fig. 1] in the absence of single B-factors gave results almost identical with those described for *Tribolium*.

Silvanus surinamensis sometimes gave erratic results which could not be attributed to dietary factors. In the absence of aneurin, pyridoxin,

choline chloride, inositol or *p*-aminobenzoic acid, growth was not impaired. Absence of riboflavin caused high mortality and considerable delay in growth, and without nicotinic acid or pantothenic acid hardly any growth took place.

Effects of choline, inositol and p*-aminobenzoic acid*

To obtain a clear indication as to the nutritional importance of choline, inositol and *p*-aminobenzoic acid, a series of tests was devised for *Tribolium* and *Ptinus*, in which the diets contained these substances in various combinations. Diet no. 6, Table 2, was used. With *Tribolium* no difference was found in the efficiency of diets containing all the vitamins compared with diets lacking either inositol, or *p*-aminobenzoic acid, or both. Without choline chloride, the diet became considerably inferior, much more so than in the corresponding tests of Fig. 2. Pupation started after 37 days, as compared with 30 days on the 'all vitamins' diet. Omission of inositol + choline, or *p*-aminobenzoic acid + choline or of all three substances had no effect beyond that shown by the absence of choline.

With *Ptinus* the result was somewhat different (Fig. 3). Omission of inositol, or *p*-aminobenzoic

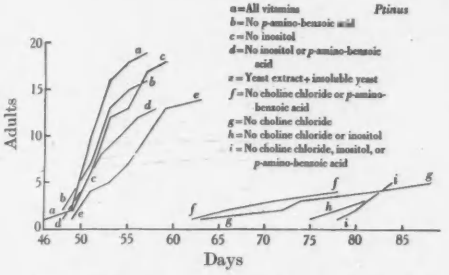
a = All vitamins
b = No p-amino-benzoic acid
c = No inositol
d = No inositol or p-amino-benzoic acid
e = Yeast extract + insoluble yeast
f = No choline chloride or p-amino-benzoic acid
g = No choline chloride
h = No choline chloride or inositol
i = No choline chloride, inositol, or p-amino-benzoic acid
Ptinus
Adults
Days

Fig. 3. *Ptinus tectus*. Explanations as for Fig. 2.

acid singly had a slight, but possibly not significant delaying effect, while a cumulative effect of the absence of both these substances became manifest. Without choline chloride, the diet became very inefficient. Omission of *p*-aminobenzoic acid + choline had no further effect, but without choline + inositol the diet was inferior to the 'no choline' diet. From these tests it appears that inositol is of some nutritional value for *Ptinus*.

Biotin as an insect growth factor

In the experiments so far described, with one exception (p. 688), the diets contained the insoluble fraction of yeast. Earlier experiments [Fraenkel & Blewett, 1943*b*] had shown that the water-soluble fraction of yeast was not fully equivalent to whole yeast, but had to be supplemented by the insoluble fraction, which was eventually found to be effective because it supplied biotin. The initial steps in the analysis which led to the recognition of biotin as the missing factor are described elsewhere [Fraenkel & Blewett, 1942*a*]. The result of a test with crystalline biotin methyl ester (25 μg. dissolved in 1 ml. of ethanol) is given in Fig. 4. The solution was taken up in a 1 ml.

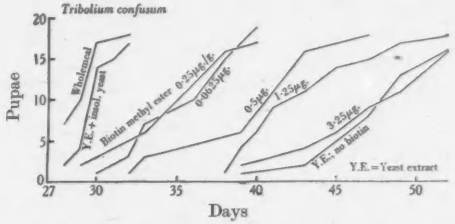

Fig. 4. Growth of *Tribolium confusum* on the yeast extract diet with the addition of graded quantities of biotin methyl ester.

hypodermic syringe and the quantities required were pipetted through the needle into the diets. The yeast extract diet was greatly improved by adding biotin methyl ester in quantities corresponding to 0·25 and 0·0625 μg./g. dry diet. In larger quantities biotin had a toxic effect. Quantities lower than 0·0625 μg./g. were not tested in this series. This very small dose produced an optimal effect which at first suggested that the optimal dose might be lower still. The toxic effects of larger quantities were not due to insufficient removal of the alcohol.

The experiment recorded in Fig. 4 was subsequently repeated with pure biotin in place of the methyl ester (Fig. 5). Growth was appreciably

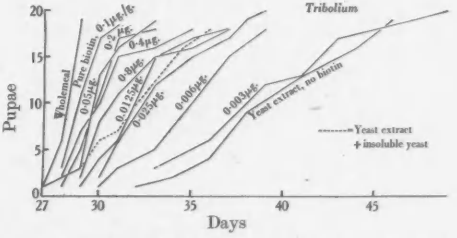

Fig. 5. Growth of *Tribolium confusum* on the yeast extract diet with the addition of graded quantities of biotin.

faster on the yeast extract diet without biotin than it had been on the former occasion. This is considered to be due to traces of biotin in the yeast extract of the Fig. 5 series. The optimum concentration of biotin was reached with 0·1 μg./g. dry food, 0·05 μg./g. being almost as efficient. Larger

quantities again appeared to be toxic, while lower quantities show a graded decreasing response with the threshold at the very low quantity of between 0·003 and 0·006 μg./g. This does not exclude the possibility that an optimal effect would have been shown with 0·1 μg. biotin methyl ester.

The difficulty of standardizing the yeast extract and getting consistent results made it desirable to construct a biotin-free diet which contained the vitamins of the B-complex as pure substances, instead of in yeast extract. The results of this series are given on p. 688 [cf. also Fraenkel & Blewett, 1943*a*, Fig. 3]. This diet, containing no yeast fractions, but 0·1 μg. biotin/g. of dry diet, is clearly as efficient as a diet which contains insoluble yeast as a source of biotin. Leaving out wheat-germ oil did not alter the efficiency of the diet, but without biotin growth became much slower and the efficiency of the biotin-free diet was about that of a typical yeast extract diet without biotin. The results of these tests showed that for *Tribolium* biotin is the only factor missing in a diet which contains yeast extract and no 'insoluble yeast'.

exception of *Silvanus*, omission of 'insoluble yeast' causes the diet to become deficient, and that the deficiency is made good by adding biotin.

A few tests were performed with the diaminocarboxylic acid which results from hydrolysis of the urea ring of biotin [du Vigneaud, 1942] and is capable of stimulating the growth of yeast in a biotin-free medium, exhibiting about 10% of the activity of biotin [du Vigneaud, Dittmer, Hoffmann & Melville, 1942]. For *Tribolium*, a biotin-free diet was considerably improved by the addition of 2·5 or 5·25 μg./g. of this diaminocarboxylic acid, but still remained considerably inferior to the same diet with biotin (0·1 μg./g.) added. 1 μg./g. or less of the diaminocarboxylic acid in the diet had no effect whatsoever (Fig. 6). These tests show clearly that biotin diaminocarboxylic acid has some effect in replacing biotin for *Tribolium*, but that, as with yeast, its activity is considerably less than that of biotin. This lesser activity cannot be compensated by using larger doses, because of a toxic effect.

All the tests so far described used diets containing glucose. On a yeast extract diet containing

Table 4. *Medium growth rate, period of development and number of pupae* (Tribolium) *or adults* (*other insects*) *formed, in the presence or absence of insoluble yeast or biotin*

(Explanation as for Table 3.)

	Tribolium			*Ptinus*			*Silvanus*			*Lasioderma*			*Sitodrepa*		
Diet includes	Growth rate	Days	Nos.	Growth rate	Days	Nos.	Growth rate	Days	Nos.	Growth rate	Days	Nos.	Growth rate	Days	Nos.
Yeast extract + 'insoluble yeast'	100	29–36	18	100	47–58	18	100	27–35	16	100	34–39	19	100	37–41	17
Yeast extract + biotin	107	28–32	19	92	54–70	16	95	28–35	16	92	26–39	17	100	37–39	15
Yeast extract alone	80	32–46	19	83	56–70	15	95	29–36	14	69	41–64	14	91	39–51	13

Concurrently with these tests with *Tribolium*, corresponding tests were performed with four other beetles. The results are summarized in Table 4. It

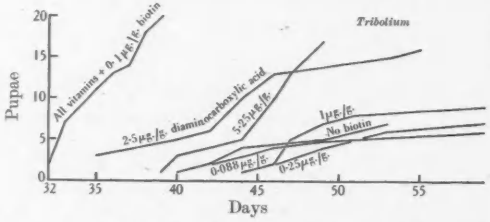

Fig. 6. Growth of *Tribolium confusum* on diets consisting of casein, glucose, cholesterol, salts, the eight members of the vitamin B-complex listed in Table 2, and in the presence or absence of biotin, or of graded amounts of 'biotin' diaminocarboxylic acid.

is clear [cf. also Fraenkel & Blewett, 1943*b*, Figs. 7–11 and Table 2] that for all the insects, with the

starch instead of glucose, *Tribolium* grew much better than in the diet containing glucose. The starch diet was almost as efficient as if it had contained 'insoluble yeast'. The effect was traced to the fact that even purified starch contained enough biotin to allow almost optimal growth of *Tribolium*.

DISCUSSION

A comparison of the effects of the absence of single B-vitamins on the different insects is given in Table 3. The most striking result is the difference in the nutritional requirements of *Tribolium* and *Ptinus* on the one hand, and *Silvanus*, *Lasioderma* and *Sitodrepa* on the other. The vitamin requirements of *Tribolium* and *Ptinus* are similar to those of the rat, dog and chick. Broadly speaking, aneurin, riboflavin, nicotinic acid, pyridoxin and pantothenic acid are indispensable; in their absence growth is greatly affected. Choline and biotin are of somewhat lesser,

but still great importance, whereas inositol and *p*-aminobenzoic acid are of minor or perhaps no importance. With the other insects, the above-mentioned 'indispensable' vitamins seem to be less important, although their requirements proved very different in detail.

These two groups of insects differ in one important anatomical and physiological feature. *Lasioderma*, *Sitodrepa* and *Silvanus* belong to the large group of insects which contain intracellular symbionts [Buchner, 1930; Koch, 1933 *a*, *b*; 1936]. The latter seem to be absent with *Tribolium* and *Ptinus*. From the distribution of intracellular symbioses in insects several authors have considered their function to be provision of accessory food substances: Wigglesworth [1929], Buchner [1930], Aschner [1931], and Koch [1933 *a*, *b*] produced evidence in support of this suggestion for *Sitodrepa panicea*. This insect when freed of its symbionts will not grow on 'Erbswurst', an otherwise satisfactory food, but will grow on 'Erbswurst' plus 25% yeast. Our results reported in this paper strongly support the view that intracellular symbionts are sources of vitamins. In their absence, with *Tribolium* and *Ptinus*, the 'indispensable' vitamins are necessary; in their presence *Lasioderma* grows well without any of them, *Silvanus* can do without aneurin and pyridoxin and *Sitodrepa* without nicotinic acid, pyridoxin and riboflavin. More recent experiments have shown that *Sitodrepa* and *Lasioderma*, when grown in the absence of symbionts, have vitamin requirements which are much more exacting than those of the normal larvae and resemble in every respect those of *Ptinus* and *Tribolium* [Fraenkel & Blewett, 1943*d*; 1944]. But even with *Tribolium* and *Ptinus*, in the absence of some of the 'indispensable' vitamins some growth takes place, and this may be due to partial intestinal synthesis. A parallel case is the synthesis of aneurin, riboflavin, nicotinic acid, pyridoxin, pantothenic acid and biotin by micro-organisms in the stomach of ruminants [Wegner, Booth, Elvehjem & Hart, 1941; McElroy & Goss, 1941]. Intestinal synthesis of biotin in the rat has been demonstrated by Nielsen & Elvehjem [1942] and by Daft, Ashburn & Sebrell [1942], who produced symptoms of a deficiency, curable with biotin, by feeding rats with the bactericidal drugs sulphanilylguanidine and succinyl sulphathiazole.

Insects require essentially the same factors of the vitamin B-complex as rats, chicks and micro-organisms. *Drosophila* requires aneurin, riboflavin [van t' Hoog, 1935, 1936], nicotinic acid, pyridoxin and pantothenic acid [Tatum, 1939, 1941]; mosquitoes require the same substances [Trager & Subbarow, 1938; Subbarow & Trager, 1940]; *Tribolium* requires aneurin, riboflavin [Fröbrich, 1939; Offhaus, 1939], and nicotinic acid [Barton-Wright, 1941]; *Galleria* requires nicotinic acid [Rubinshtein & Shekun, 1941]. In none of these cases did the insects grow satisfactorily without the further addition of yeast or yeast fractions. Insects have also been shown to require for optimal growth in addition to yeast the water-insoluble fraction of yeast (Hobson [1933] for blowfly larvae, Fröbrich [1939] for *Tribolium*, and Tatum [1939, 1941] for the *Drosophila* larva). The need of *Tribolium* for biotin [Fraenkel & Blewett, 1942*b*] has been confirmed by Rosenthal & Reichstein [1942].

The need of certain insects for choline chloride now appears clear. In tests with *Tribolium*, omission of choline definitely decreases the efficiency of the diet. The need for choline was still more marked in experiments with *Ptinus* (Fig. 3) and most strikingly demonstrated with *Lasioderma* [Fraenkel & Blewett, 1943*a*, Fig. 2], in which choline seems to be the most important of the B-vitamins. With inositol and *p*-aminobenzoic acid the position is less clear and the results are conflicting, a position which closely resembles that experienced with vertebrates [György, 1942].

For *Tribolium* a diet without any yeast, but with the water-soluble B-vitamins and biotin in pure substance, with cholesterol and without wheat-germ oil, is in every respect as good as one with yeast or yeast fractions. This would appear to show that no further factors are required. *Ptinus*, *Sitodrepa* and *Lasioderma* also grow about equally fast with either insoluble yeast or biotin. All the insects, with the exception of *Sitodrepa*, grow as well on mixtures of pure B-vitamins as on yeast extract. Taking all the evidence together, biotin and sterols seem to be the only water-insoluble factors in yeast which are required by the insects in question. Also they do not seem to require any B-vitamins in addition to those already discovered (exception, *Sitodrepa*).

SUMMARY

1. Five insect species have been grown on an artificial diet which contains all the chemically identified members of the vitamin B-complex in pure form. For *Tribolium* and *Ptinus*, aneurin, riboflavin, nicotinic acid, pyridoxin and pantothenic acid are essential vitamins; choline chloride is certainly, and inositol and *p*-aminobenzoic acid are possibly, of importance as growth factors.

2. *Lasioderma*, *Sitodrepa* and *Silvanus* do not seem to require some of the vitamins which are indispensable for *Tribolium* and *Ptinus*. This difference in the vitamin requirements is due to the presence in *Lasioderma*, *Sitodrepa* and *Silvanus* of intracellular symbiotic micro-organisms, which synthesize some of the vitamins.

3. When bred on a diet of casein, glucose, cholesterol, salts and aqueous yeast extract, insects

require, in addition, for optimal growth a substance contained in the water-insoluble residue of yeast, which was found to be biotin.

4. The optimal requirements of biotin for *Tribolium* are approximately 0·1 μg./g. of the dry diet, and the threshold of action is between 0·003 and 0·006 μg./g.

5. The insects tested do not appear to require any water-insoluble factor present in yeast in addition to a sterol and biotin.

6. The insects used, with the exception of *Sitodrepa*, grow on a mixture of pure B-vitamins as well as on yeast extract. It is concluded that these insects do not require B-vitamins beyond those already isolated.

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REFERENCES

Aschner, M. [1931]. *Z. Morph. Ökol. Tiere*, **20**, 368.
Barton-Wright, E. [1941]. *Nature, Lond.*, **148**, 565.
Buchner, P. [1930]. *Tiere und Pflanze in Symbiose*. Berlin: Gebrüder Borntträger.
Chick, H. & Roscoe, M. H. [1930]. *Biochem. J.* **24**, 105.
Craig, R. & Hoskins, W. M. [1940]. *Ann. Rev. Biochem.* **9**, 617.
Daft, F. S., Ashburn, L. L. & Sebrell, W. H. [1942]. *Science*, **96**, 321.
El Sadr, M. M., Macrae, T. F. & Work, C. E. [1940]. *Biochem. J.* **34**, 601.
Fraenkel, G. & Blewett, M. [1942*a*]. *Nature, Lond.*, **149**, 301.
—— —— [1942*b*]. *Nature, Lond.*, **150**, 177.
—— —— [1943*a*]. *Nature, Lond.*, **151**, 703.
—— —— [1943*b*]. *J. exp. Biol.* **20**, 28.
—— —— [1943*c*]. *Trans. ent. Soc. Lond.* **93**, 457.
—— —— [1943*d*]. *Nature, Lond.*, **152**, 506.
—— —— [1944]. *Proc. roy. Soc.* B (in the Press).
Fröbrich, G. [1939]. *Z. vergl. Physiol.* **27**, 335.
Gay, F. J. [1938]. *J. exp. Zool.* **79**, 93.
György, P. [1942]. *Ann. Rev. Biochem.* **11**, 309.
Hobson, R. P. [1933]. *Biochem. J.* **27**, 1899.
van t' Hoog, E. G. [1935]. *Z. Vitaminforsch.* **4**, 300.
—— [1936]. *Z. Vitaminforsch.* **5**, 118.
Koch, A. [1933*a*]. *Biol. Zbl.* **53**, 199.
—— [1933*b*]. *Verh. dtsch. zool. Ges.* p. 143.
—— [1936]. *Z. Morph. Ökol. Tiere*, **32**, 137.
McElroy, C. W. & Goss, H. [1941]. *J. Nutrit.* **20**, 527; **21**, 163, 405.
Nielsen, E. & Elvehjem, C. A. [1942]. *J. biol. Chem.* **145**, 713.
Offhaus, K. [1939]. *Z. vergl. Physiol.* **27**, 384.
Rosenthal, H. & Reichstein, T. [1942]. *Nature, Lond.*, **150**, 246.
Rubinshtein, D. L. & Shekun, L. A. [1941]. *Bull. biol. med. exp. U.S.S.R.* **9**, 190.
Subbarow, Y. & Trager, W. [1940]. *J. gen. Physiol.* **23**, 561.
Tatum, E. L. [1939]. *Proc. nat. Acad. Sci., Wash.*, **25**, 490.
—— [1941]. *Proc. nat. Acad. Sci., Wash.*, **27**, 193.
Trager, W. [1941]. *Physiol. Rev.* **21**, 1.
—— & Subbarow, Y. [1938]. *Biol. Bull. Woods Hole*, **75**, 75.
du Vigneaud, V. [1942]. *Science*, **96**, 455.
—— Dittmer, K., Hoffmann, K. & Melville, D. B. [1942]. *Proc. Soc. exp. Biol., N.Y.*, **50**, 374.
Wegner, M. I., Booth, A. N., Elvehjem, C. A. & Hart, E. B. [1941]. *Proc. Soc. exp. Biol., N.Y.*, **45**, 769.
Wigglesworth, V. B. [1929]. *Parasitology*, **21**, 288.

The Sterol Requirements of Several Insects

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It has been shown [Fraenkel & Blewett, 1943*a*] that six insects which normally feed on flour and other stored food grow satisfactorily on a diet of casein 50, glucose or starch 50, yeast 5, cholesterol 1, salts 1 and water 15 parts. Without cholesterol, growth became slightly (*Tribolium, Silvanus, Lasioderma*), appreciably (*Ptinus*) or severely (*Sitodrepa, Ephestia*) impaired (Table 2 (1)). If the diet contains aqueous yeast extract in place of yeast together with cholesterol, all the insects develop, though at a reduced rate resulting from a deficiency of biotin. Without cholesterol the diet becomes entirely or almost entirely inefficient for all the insects tested (Table 2 (3) and Fraenkel & Blewett [1943*a*]). In the present work the specificity of the sterol requirements of these insects is investigated.

EXPERIMENTAL

A convenient method of preparing a sterol-deficient diet is Soxhlet extraction of wholemeal flour with chloroform for at least 10 hr. Extraction with ether (Soxhlet) proved unsuccessful in removing the sterols sufficiently. Addition of various sterols fully restored the nutritional qualities of the flour. This effect was used as a means of comparing the nutritional values of sterols and sterol derivatives. In addition to the insects enumerated by Fraenkel & Blewett [1943*b*, p. 686], the flour moth *Ephestia kuehniella* Zell. was also used. The methods of experimenting and assessing results are fully described elsewhere [Fraenkel & Blewett, 1943 *a*, *c*].

The following sterols and sterol derivatives were used:

Cholesterol, sitosterol, ergosterol, calciferol, 7-ketocholesteryl acetate, 7-hydroxycholesterol, 7-hydroxycholesteryl dibenzoate, 7-dehydrocholesteryl monobenzoate, 7-dehydrocholesterol, cholestanol free from cholesterol (Glaxo Laboratories Ltd.).

Cholesterol purified via the dibromide, cholestanol (dihydrocholesterol), zymosterol, Δ^5-cholestene, cholesteryl acetate (Prof. I. M. Heilbron and Dr E. R. H. Jones).

RESULTS

On wholemeal flour extracted with chloroform, *Sitodrepa* and *Ephestia* did not grow at all, *Ptinus* grew very slowly with only very few insects completing development, while *Tribolium*, *Lasioderma* and *Silvanus* grew somewhat better but still at a greatly reduced rate. Adding a few drops of the chloroform extract to extracted flour restored it to almost its original efficiency. Wheat-germ oil (Vitamins Ltd.) restored extracted flour to its full nutritional value for *Tribolium* and *Ephestia* and was more efficient than the chloroform extract (Table 1 and Figs. 1, 2).

Table 1. *Growth on flour extracted with chloroform and with the addition of the extracted material or a number of sterols or sterol derivatives*

	Growth						
	With basal diet of $CHCl_3$-extracted wholemeal flour, of						With basal diet of yeast and fructose, of
Addition to basal diet	*Tribolium*	*Lasioderma*	*Silvanus*	*Ptinus*	*Sitodrepa*	*Ephestia*	*Dermestes*
None	+	+	+ +	±	−	−	−
Chloroform extract	+ + + +	+ + + +	+ + + +	+ + +	+ + +	+ + +	−
Wheat-germ oil	+ + + +	.	.	.	.	+ + + + +	.
Cholesterol	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+ + + +
Sitosterol	+ + + +	+ + + +	+ + + +	+ + +	+ + + +	+ + +	−
Ergosterol	+ + + +	+ + + +	+ + +	+ + + +	+ + + +	+ +	−
Cholestanol	+ + (+)	+ +	+ + +	+ +	+ + +	+	−
Δ^5-Cholestene	+ (+)	.	.	.	−	−	−
Zymosterol	+ (+)	+ +	+ + +	+ (+)	−	−	−
Calciferol	−	±	+ +	−	−	−	−
7-Dehydrocholesterol	+ + + +	+ + + +	+ + + +	+ + + +	+ + + +	+ + +	+ + + +
Cholesteryl-acetate	+ + + +	.	.	.	+ + + +	+ + +	+ + + +
7-Hydroxycholesterol	±	.	.	.	−	−	−
7-Dehydrocholesteryl monobenzoate	+ +	.	.	.	−	−	+ + + +
7-Hydroxycholesteryl dibenzoate	±	.	.	.	−	−	−
7-Ketocholesteryl acetate	−	.	.	.	−	−	−

The + and − signs give a rough quantitative estimate of the relative efficiency of diets. Explanation of the symbols in Fraenkel & Blewett [1943*a*].

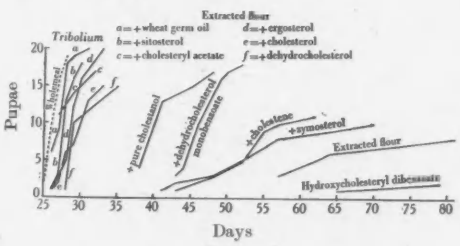

Fig. 1.

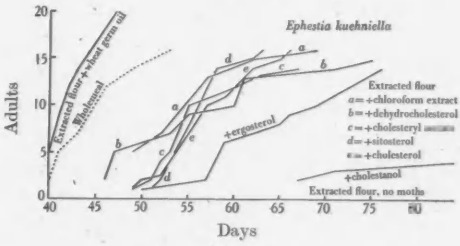

Fig. 2.

Fig. 1. *Tribolium confusum*. Growth on wholemeal flour extracted with chloroform, and on extracted flour with the addition of wheat-germ oil, sterols or sterol derivatives.

Fig. 2. *Ephestia kuehniella*. Explanation as for Fig. 1.

The results of adding sterols and sterol derivatives to extracted flour are summarized in Table 1, and are given for *Tribolium* and *Ephestia* in Figs. 1 and 2. Adding cholesterol, ergosterol or sitosterol restored its original efficiency either entirely or almost entirely. Only with *Ephestia* wheat-germ oil proved more efficient than any single sterol (Fig. 2) and from these results, and others not yet published, it seems likely that other fat-soluble factors, possibly unsaturated fatty acids, are required by *Ephestia*. Destruction of such unknown factors during the prolonged chloroform extraction at a high temperature may explain why wheat-germ oil proved more efficient for *Ephestia* than the chloroform extract. We do not yet know whether small differences in the efficiency of cholesterol, ergosterol or sitosterol are significant. Of the other sterols tested, only 7-dehydrocholesterol and cholesterylacetate proved of about the same nutritional value as the three common natural sterols.

In evaluating the effect of sterols and sterol derivatives it has to be borne in mind that extracted flour proved entirely inefficient only for *Sitodrepa* and *Ephestia*, while some growth took place with the other species. This growth was almost certainly only possible through traces of sterols remaining in the flour after extraction. Furthermore, most of the derivatives of cholesterol which we tested were not pure and almost certainly contained cholesterol, while zymosterol was probably not free from ergosterol. Traces of any physiologically active sterol would, therefore, be expected to improve further the extracted flour for those insects which already showed some growth on it by virtue of small amounts of sterol retained. The insects which required large amounts of sterol, and which entirely failed to grow on extracted flour, viz. *Ephestia* and *Sitodrepa*, grew well only on cholesterol, sitosterol, ergosterol, cholesteryl acetate and 7-dehydrocholesterol, and, to a lesser degree, on cholestanol (Fig. 2). From this it appeared probable that the seemingly positive effects of zymosterol on *Lasioderma* and *Silvanus*, and of Δ^5-cholestene, dehydrocholesterol monobenzoate and zymosterol on *Tribolium*, were due to impurities. This does not apply to cholestanol, of which a pure sample was obtained on which even *Sitodrepa* and *Ephestia* grew fairly well. Cholestanol is, therefore, of some food value, although inferior to the 'good' sterols. Calciferol (vitamin D_2) was not utilized by any of the insects tested.

DISCUSSION

It has been known for several years that sterols are growth factors for certain insects (literature in Fraenkel, Reid & Blewett [1941]) and this is now shown to be true for six more insects. It is also now certain that the fat-soluble factor necessary for *Ephestia kuehniella*, described by Richardson [1926] as vitamin A, is in fact a sterol. The last column of Table 1 contains the results of a previous investigation of the sterol requirements of another beetle, *Dermestes vulpinus* [Fraenkel *et al.* 1941]. The striking difference in the sterol requirements of *Dermestes* and the flour insects is that *Dermestes* will not grow on ergosterol or sitosterol. This limits its distribution to food of animal origin, while the flour insects grow equally well on a diet of vegetable origin (containing sitosterol), animal origin (containing cholesterol) or micro-organisms (containing ergosterol) [see Fraenkel *et al.* 1941; Fraenkel & Blewett, 1943*c*].

The present work confirms the observation that certain insects require sterols in the diet, and not calciferol (vitamin D_2), in which the tetracyclic arrangement of the carbon atoms has been changed and which is consequently not a true sterol. 7-Dehydrosterols are utilized as well as sterols with only one double bond in ring B (cholesterol, sitosterol). There is, however, no reason to assume that cholesterol and sitosterol are utilized by virtue of traces of the respective dehydrosterols which are usually associated with them in biological material. Cholesterol, purified via the dibromide and free of dehydrocholesterol, proved just as efficient as unpurified cholesterol. Of the cholesterol derivatives, only the acetic ester retained full activity, and dihydrocholesterol (cholestanol) appeared of some food value. All the other structural changes, such as loss of the secondary alcohol group in Δ^5-cholestene, introduction of a second alcohol group in 7-hydroxycholesterol, and esterification of this group in 7-hydroxycholesteryl dibenzoate or introduction of a keto-group on carbon atom 7 in 7-ketocholesteryl-acetate, caused loss of nutritional value.

Table 2. *Growth on diets slightly* (1) *or grossly* (2 *and* 3) *deficient in sterols*

Diet	Growth of *Tribolium*	*Lasioderma*	*Silvanus*	*Ptinus*	*Sitodrepa*	*Ephestia*
(1) Casein, starch, 5% yeast	+ + +	+ + +	+ + +	+ +	±	–
(2) Flour extracted with chloroform	+	+	+ +	±	–	–
(3) Casein, glucose, yeast extract	+	+	+	–	–	–

The requirements for sterols seem, therefore, to be qualitatively the same for the six insect species under investigation, but there are obvious quantitative differences. From a comparison of the growth on three diets relatively deficient in sterols (summarized in Table 2) it can be concluded that *Sitodrepa*, *Ptinus* and *Ephestia kuehniella* require larger quantities of sterols than *Tribolium*, *Lasioderma* and *Silvanus*.

SUMMARY

The sterol requirements of six different insect species have been investigated; all the insects require a sterol in the diet for growth. The quantitative requirements of sterols by different insects are different, but the qualitative requirements of the insects under investigation are the same. The specificity of the sterol requirements was studied.

We gratefully acknowledge a Government grant from the Royal Society, which, partly, made this investigation possible. We are also much indebted to Prof. I. M. Heilbron and Dr E. R. H. Jones (Imperial College of Science and Technology) and to Mr A. L. Bacharach and Dr E. Lester Smith (Glaxo Laboratories Ltd.) for the gift of a number of sterols.

REFERENCES

Fraenkel, G. & Blewett, M. [1943*a*]. *J. exp. Biol.* **20**, 28.
—— —— [1943*b*]. *Biochem. J.* **37**, 686.
—— —— [1943*c*]. *Trans. R. ent. Soc.* **93**, 457.
Fraenkel, G., Reid, J. A. & Blewett, M. [1941]. *Biochem. J.* **35**, 712.
Richardson, C. H. [1926]. *J. agric. Res.* **32**, 895.

Apparent Vitamin C in Foods

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Titration with 2:6-dichlorophenolindophenol is used to estimate vitamin C in a wide variety of foodstuffs, though the dye may react with substances besides ascorbic acid. Harris [1933] showed that dye-reducing substances can be produced by heating certain sugars with alkali. Johnson [1933] found with germinated peas that dye titrations indicated higher vitamin C contents than was shown by biological tests. In acid extracts part of the dye-reducing power was due to a substance which reduced the dye more slowly than ascorbic acid. Bacharach, Cook & Smith [1934] found an increase in the dye-reducing power of malt extract heated to 100° for ½–2 hr. McHenry & Graham [1935] obtained increases of 50–170% by heating certain vegetables for a few minutes at 58° or by standing these for a few hours at room temperature in 1% HCl. Ahmad [1935] found that boiling cabbage for 10 min. with 20% CCl_3COOH increased the apparent vitamin C content by nearly 200%. Reedman & McHenry [1938] found that the apparent vitamin C in heated potatoes resembled true vitamin C in both physiological and many chemical properties. Their suggestion that ascorbic acid might exist in combination with protein from which it was set free by heating was discredited by Harris & Olliver [1942], who suggested three possible causes for the results: (1) incomplete extraction from unheated tissues, (2) variations in sampling, (3) inhibition by heat of the ascorbic acid oxidase in the foods examined. The possible occurrence of non-specific dye-reducing substances in vegetable tissues was considered of little or no practical importance, although it was admitted that germinated seedlings, malt extract, yeast and fermented juices might repay further study.

During the last three years we have been accumulating evidence to show that the apparent vitamin C content of foods can become considerably higher than the true vitamin C value under manufacturing and storage conditions which may occur in practice. Preliminary reference to these findings was made by Wokes, Johnson, Duncan, Organ & Jacoby [1942] and by Wokes, Organ, Duncan & Jacoby [1943*a*]. The provisional term 'apparent vitamin C' was proposed to describe substances occurring in natural and processed foods which, although not possessing antiscorbutic activity, simulate vitamin C very closely in their chemical and physical properties, so that they are not distinguished from it by the indophenol titration as usually applied, and may therefore cause results to be too high. Methods of estimating true vitamin C in the presence of this apparent vitamin C have

been described by Mapson [1943*a*,*b*] and by Wokes, Organ & Jacoby [1943*b*]. In this paper a brief account is given of the production and occurrence of apparent vitamin C in foodstuffs, and of some of its properties.

EXPERIMENTAL

Indophenol titrations

In our earlier experiments we used only the visual dye titration method essentially as described by Harris & Olliver [1942]. Later we also used the potentiometric method of Harris, Mapson & Wang [1942] with modifications. This proved invaluable both for deeply coloured products and for following dye reactions more precisely than is possible visually, and we are greatly indebted to these workers for details of the method prior to publication. Satisfactory agreement was obtained between visual and potentiometric results, provided that timing and rate of dye additions were carefully controlled as described by Wokes *et al.* [1943*b*].

Estimation of apparent vitamin C

This was carried out by a modification [Wokes *et al.* 1943*b*] of the method of Lugg [1942]. Lugg divided the various dye-reducing substances into three groups according to their rate of reaction with formaldehyde: (*a*) react readily at pH 1·5 and 3·5, (*b*) react readily at pH 3·5, slowly at pH 1·5, (*c*) do not react readily at either pH. Ascorbic acid was the only substance he had found in group (*b*); reductone occurred in group (*c*). Hence a basis was provided for differentiating ascorbic acid from reductone.

It has been shown by Mapson [1943*b*] and ourselves [Wokes *et al.* 1943*b*] that reductone and other components of apparent vitamin C react too rapidly with formaldehyde at pH 3·5 for satisfactory differentiation. We have obtained satisfactory results by treating mixtures of true and apparent vitamin C with formaldehyde at pH 4·5 for 6 min. This destroys the whole of the true vitamin C but little, if any, of the apparent vitamin C. Recovery experiments are made to correct for any apparent vitamin C destroyed in certain materials. The action of the formaldehyde is checked at the end of 6 min. by adding excess HPO_3 to bring the pH to about 1·0. To calculate true vitamin C the apparent vitamin C thus determined is subtracted from the total vitamin C estimated with similar timing and rate of dye additions.

Production of apparent vitamin C by heating with mineral acid

Potatoes, cabbages, carrots, germinated grains (wheat, barley and oats), grass and lucerne were treated with various concentrations of HCl, H_2SO_4, HPO_3 or CCl_3COOH in a water-bath at 90–95°, or occasionally on a sand-bath, water being added at intervals to make good any loss. With some materials (e.g. grass), heating on a sand-bath proved more effective. With HCl or H_2SO_4, 2% (v/v) of the concentrated acid gave the best results, producing as much apparent vitamin C as higher (up to 8%) concentrations of acid, but when less than 2% of concentrated acid was used the rate of production of apparent vitamin C fell rapidly with decreasing acid concentration. 1% HPO_3 was more effective than lower concentrations, but the rate of production of apparent vitamin C was still much slower than with 2% conc. HCl. The apparent vitamin C produced with 1% HPO_3 or with 0·25% conc. HCl was unstable to prolonged heating. When heating cabbage extract in 25% CCl_3COOH we were not able to obtain any increase in the dye titration values such as Ahmad [1935] described, but, on the contrary, as heating continued the titration value became less. Our cabbage extracts were prepared by grinding with quartz sand, with precautions to avoid destruction of ascorbic acid by oxidizing enzymes [vide Wokes & Organ, 1943]. Hence our findings indicate that the increase in the dye titration value observed by Ahmad when heating cabbage with 25% CCl_3COOH was due to extraction difficulties and not to production of apparent vitamin C. We were able to find only slight traces of apparent vitamin C in our cabbage extracts when examining these by the method of Wokes *et al.* [1943*b*]. On the other hand, when we heated germinated grains, grass and lucerne with acid, the dye titration figures steadily increased during several hours' heating (in contrast with the increase in 10 min. observed by Ahmad). The presence of apparent vitamin C was confirmed by the above method, although the amount found was sometimes less than the increase observed, and the possibility of formation of hydroxytetronic acid was not precluded. Nevertheless, ample evidence was obtained that the increase in the dye titration values was not due to any of the three possible causes mentioned by Harris & Olliver, viz. incomplete extraction, variation in sampling, or inhibition of ascorbic acid oxidase. A few representative results are given in Fig. 1.

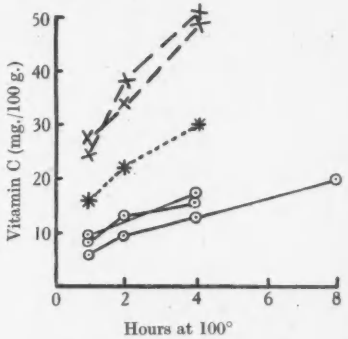


Fig. 1. Production of apparent vitamin C by heating with 2% conc. HCl or of conc. H_2SO_4 at 100°. Germinated grains, ⊙——⊙. Grass, ×——×. Lucerne, ✳- - - - -✳.

Production of apparent vitamin C by heating without acid

In certain foods and food materials apparent vitamin C can be produced by heating to about 100° without addition of acid. This effect is strongly marked with malt extract, in which it is possible to increase the apparent vitamin C content as much as 10 times, but may also be observed with fruit syrups and other products. If true vitamin C be present, its destruction by heat may be masked by the simultaneous production of apparent vitamin C. This production of apparent vitamin C can take place at pH 4–5 as compared with a pH below 1 in the experiments with acid. A few typical results are given in Table 1.

Table 1. *Apparent and true vitamin C contents of foods, etc.*

Material	pH of material	Vitamin C content (mg./100 g.) Total	Apparent (*a*)	True	True as percentage of total
Fresh fruit and fruit preparations:					
Black currants, fresh	3·2	202	4	198	99
Black currant juice	3·2	140	5	135	96
Black currant syrup, freshly made	—	103	2	101	98
Black currant syrup after 8 weeks at room temp. in dark	—	96	4	92	96
Black currant syrup after 13 weeks at 27° in dark	—	58	3	55	94
Black currant syrup after 10 weeks at 37° in dark	—	68	8	60	88
Rose hip syrup after 12 months at 0–5°	3·7	156	20	136	88
Rose hip syrup after 12 months	3·6	101	69	32	31
Cherry juice, conc., after 5 years at 0–5°	3·8	47	45	2	4
Lemon juice, conc., after 18 months at 0–5°	1·7	193	42	151	78
Lemon juice, conc., after 4 years at 0–5°	1·7	27	21	6	22
Walnuts, unripe (*Juglans regia*), 2–3 g.	—	2619	1310	1309	50
Walnuts, unripe, 6–7 g.	5·2	2961	987	1974	67
Walnuts, unripe, 15–20 g.	3·9	2028	408	1620	80
Walnuts, unripe, after precipitation of tannin (*b*)	3·9	1909	400	1509	79
Dried fruits and vegetables:					
Dried bananas (*c*)	5·0	6	1	5	83
Dried carrots (*c*)	4·8	90	49	41	45
Dried potatoes (*d*)	4·5	27	4	23	85
Dried rose hip extract (mean of five samples) (*e*)	4·1	1300	50	1250	96
Dried spinach (*c*)	7·0	2	0·5	1·5	75
Dried tomatoes (*c*)	4·7	89	55	34	38
Malt extracts:					
Liquid malt extract, low diastatic power (D.P.)	4·7	3	(*f*)	0	0
Liquid malt extract, normal D.P.	4·7	12	(*f*)	0	0
Liquid malt extract, normal D.P.	4·8	5	(*f*)	0	0
Liquid malt extract after ½ hr. at 100°	4·8	12	(*f*)	0	0
Liquid malt extract after 2 hr. at 100°	4·8	20	(*f*)	0	0
Liquid malt extract after 9 months at 37°	3·6	103	(*f*)	0	0
Dried malt extract, low D.P. (*g*)	4·7	3	(*f*)	0	0
Dried malt extract, normal D.P. (*g*)	4·7	12	(*f*)	0	0
Sugars and molasses:					
Sugar, white (mean of three samples)	—	0	0	0	0
Sugar, brown (mean of four samples)	—	1	1	0	0
Golden syrup	—	27	27	0	0
Molasses, beet	—	18	18	0	0
Molasses, cane	—	11	11	0	0
Molasses, before charcoal treatment (*h*)	5·6	46	46	0	0
Molasses, after charcoal treatment	6·2	19	19	0	0
Molasses, beet concentrated preparation	5·4	222	(*d*)	0	0
Molasses, beet concentrated preparation	—	304	(*d*)	0	0
Molasses, cane concentrated preparation	—	196	(*d*)	0	0
Cocoa, chocolate:					
Cocoa, defatted, mixed sample	7·0	65	65	0	0
Cocoa, defatted, after precipitation of tannin	7·0	12	(*d*)	0	0
Chocolate, vitaminized	6·1	44	8	36	82
Chocolate, vitaminized	5·7	36	4	32	89
Miscellaneous:					
Beer, light draught	4·6	0·3	0·3	0	0
Beer, dark draught	4·4	1	1	0	0
Parsley (*i*)	7·0	220	40	180	82

Notes

(*a*) Apparent vitamin C determined by method of Wokes *et al.* [1943*b*] checked occasionally by Mapson's method [1943*b*].
(*b*) By method of Mirimanoff & Mori [1940].
(*c*) After about 4 years' storage in air-filled containers at room temperature.
(*d*) After 6 weeks' storage in air-filled containers at room temperature.
(*e*) pH range 4·0–4·2.
(*f*) Some of the dye reductant in these preparations reacted with HCHO under the given conditions, but since it had been produced by heating was considered not to be ascorbic acid.
(*g*) Prepared by evaporation *in vacuo* of the corresponding liquid malt extract.
(*h*) Treatment normally used in refining sugar.
(*i*) During extraction cyanide used to inhibit action of oxidizing enzymes and stabilize apparent and true vitamin C.

Development of apparent vitamin C during storage

Apparent vitamin C may be gradually formed during storage under normal conditions. The rate of formation is affected by temperature, moisture content and exposure to air and enzymic action.

(*a*) *Effect of storage temperature.* Fig. 2 summarizes some results obtained on malt extract, which show that the rate of formation of apparent vitamin C is affected by the

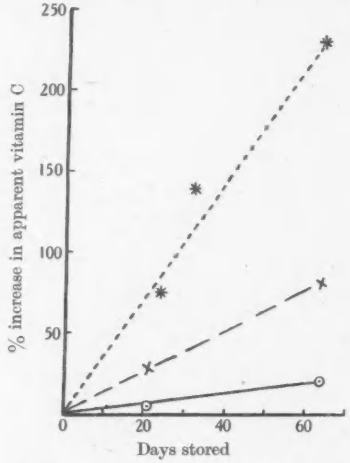


Fig. 2. Effect of storage temperature on rate of production of apparent vitamin C in malt extract. Increase at 37°, ✱-----✱. Increase at room temperature, ×——×. Increase at 0–5°, ⊙——⊙.

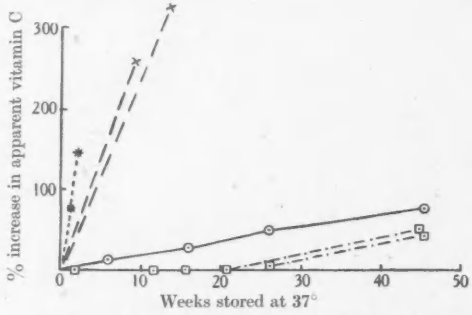


Fig. 3. Rate of production of apparent vitamin C in malt extract stored in air-filled containers at 37°, showing effect of enzyme and moisture content. Results on dried extract of low diastatic power (12 Lintner), ⊡-.-.-⊡. Dried extract of normal diastatic power (112 Lintner), ⊙——⊙. Liquid extract from which low D.P. dry extract prepared, ×——×. Liquid extract from which normal d.p. dried ext. prepared, ✱-----✱.

storage temperature. At 37° increases of 12–103 mg./100 g. were observed during 268 days' storage. In the refrigerator at about 5° an increase of 46% was observed in 150 days.

(*b*) *Effect of moisture content.* Removal of all but 1–2% of the moisture greatly retards the rate of production of apparent vitamin C. This is clearly shown by results of experiments on malt extract before and after drying. Rather less conclusive evidence was obtained, indicating that destruction of the malt enzymes also diminished the rate of production of apparent vitamin C (see Fig. 3).

In dried extracts of vegetables and fruits stored for 4 years at room temperature in air-filled containers there was marked development of apparent vitamin C, masking

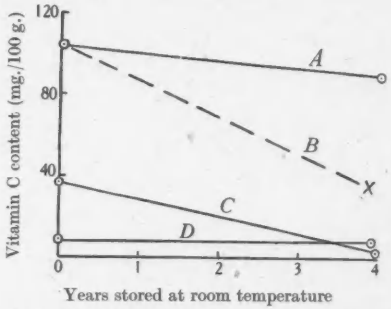


Fig. 4. Total and apparent vitamin C in dried fruit and vegetables stored in air at room temperature. *A*, total vitamin C in dried tomatoes. *B*, true vitamin C in dried tomatoes. *C*, total and true vitamin C in dried spinach. *D*, total vitamin C in dried bananas.

the gradual destruction of true vitamin C (see results with tomatoes in Fig. 4). Storage in nitrogen-filled containers reduced the rate of formation of apparent vitamin C but did not entirely prevent it.

Occurrence of apparent vitamin C in miscellaneous materials

(*a*) *Cocoa and chocolate.* Apparent vitamin C has been found in considerable amounts in cocoa, and may therefore occur in chocolate used as a carrier of the vitamin (Table 1). When the true vitamin C gradually disappears during storage, the apparent vitamin C remains, and if no correction is made for its presence the error increases in magnitude. The apparent vitamin C is not entirely tannin, since it is not completely precipitated by the method of Mirimanoff & Mori [1940].

(*b*) *Fruits and fruit syrups.* Fresh fruits appear on the whole to contain only small amounts of apparent vitamin C, which does not usually exceed 10% of the total vitamin C found. Unripe walnuts are an exception; in some species the apparent vitamin C may be 80% of the total [Melville, Wokes & Organ, 1943].

When fruit juices are heated or subjected to prolonged storage more apparent vitamin C may be produced, so that it may eventually form the greater part of the total vitamin C found by the usual dye titration (Table 1). Some of this may be reductic acid formed by the action of the fruit acids on pectin or other constituents.

(*c*) *Sugar, syrup and molasses.* Demerara and other coloured sugars contain traces of apparent vitamin C, which may produce an appreciable effect on the total vitamin C content of fruit syrups. Some samples of table syrup contain more apparent vitamin C. This is also found in molasses, from which very concentrated preparations

can be obtained. In the process of sugar refining the apparent vitamin C is presumably produced by the treatment with mineral acid, and partially removed by subsequent treatment with charcoal.

(*d*) *Vegetables.* Significant amounts of apparent vitamin C have not been found in common raw vegetables, but may be produced by cooking at a high temperature (e.g. roasting or frying), and also occur in some herbs such as sorrel and parsley.

(*e*) *Beer.* Traces of apparent vitamin C, comparable with the total vitamin C contents of South African beers reported by Levy & Fox [1935], have been found in English draught beer, especially in the dark variety.

Properties of apparent vitamin C

(*a*) *Temperature coefficient.* The rate of production of apparent vitamin C increases with rise in temperature. Between 0 and 37° the temperature coefficient for 10° rise is of the order of 2. At 100° a sharp rise in the rate occurs.

(*b*) *Effect of* pH *on rate of formation and stability.* Apparent vitamin C develops as rapidly at pH 4–5 as at pH 1, and in part at least is unstable in 5% HPO_3 solution (pH below 1) as well as in less acid aqueous solutions. Its decomposition may be retarded by bubbling nitrogen through the solution to expel air, and by storage in the dark in nitrogen-filled containers; also by the use of cyanide.

(*c*) *Effect of oxidizing enzymes.* These, including ascorbic acid oxidase, were extracted from outer cabbage leaves by the method of Szent-Györgyi [1931], from cauliflower inner florets by the method of Hopkins & Morgan [1936] and from unripe tomato skins by the method of Wokes & Organ [1943], and tested on apparent vitamin C obtained either at a pH of about 1 (e.g. beet and cane molasses, acid-heated extracts of various foods) or at a pH of about 5 (e.g. dehydrated foods, malt extract). Freshly prepared solutions in buffers of pH 2–6 were mixed with the enzyme extracts at room temperature and apparent vitamin C determined at intervals. The enzymes destroyed apparent vitamin C under the same conditions as they destroyed real vitamin C.

(*d*) *Effect of oxidizing agents.* Practically the whole of the apparent vitamin C in different foods can be destroyed within 2–3 min. by treatment with 0·1% hydrogen peroxide or sodium percarbonate at 37°. This treatment also destroys true vitamin C. With lower concentrations less of the true or apparent vitamin C was destroyed. Even $1:10^8$ dilution of H_2O_2 produced an appreciable effect. The rate of destruction was most rapid in the first minute. It did not appear possible to differentiate between true and apparent vitamin C by such means.

(*e*) *Effect of ultra-violet light.* Griffiths [1943] suggested that reductones might be formed by the action of ultra-violet light on substances in the atmosphere. Solutions of dehydrated foods, in which it was known that apparent vitamin C could be developed during prolonged storage, were irradiated (mercury vapour lamp) at pH 4–6 in covered Petri dishes for several hours, screens being used to cut out short wave-length rays not present in sunlight, without development of apparent vitamin C. Solutions of foods in which the apparent vitamin C was already present were irradiated under similar conditions, and the apparent vitamin C was gradually destroyed, having almost entirely disappeared after 3–4 hr. irradiation [Wokes, 1943]. Whilst ultra-violet light did not produce any apparent vitamin C in these dehydrated foods, the position may be quite different in natural foods, such as fruits, which are exposed to sunshine and contain various pigments and enzyme systems.

(*f*) *Spectroscopic evidence.* Dr R. A. Morton kindly examined spectroscopically three of our most concentrated sources of apparent vitamin C obtained from malt extract by prolonged incubation, and from beet and cane molasses respectively. The treatment which these had undergone precluded the presence of true vitamin C, and the absence of antiscorbutic properties was also shown by physiological tests. The incubated malt extract showed a very distinct maximum at about 280 mμ (Fig. 5). Maxima in the beet

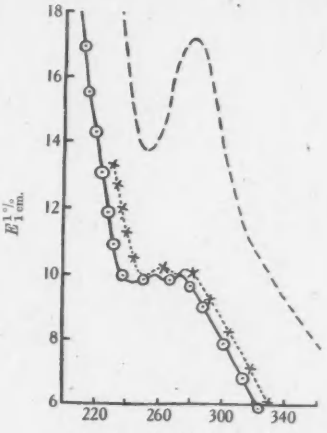


Fig. 5. Absorption spectrograms of concentrated preparations of apparent vitamin C obtained from: malt extract, ——; beet molasses, ×-----×; cane molasses, ⊙——⊙.

molasses at about 276 and 264 mμ, and in the cane molasses at about 275 and 256 mμ, were obscured by general absorption, due to the dark colour. When this dark colour was removed by treatment with charcoal, the apparent vitamin C was not recovered. Attempts to obtain colourless extracts suitable for spectroscopic examination by shaking or Soxhlet extraction with methanol or ethanol or by prolonged shaking with ether proved unsuccessful.

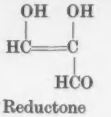
DISCUSSION

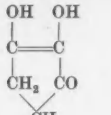
The production of non-specific dye reductants by heating different foodstuffs with or without acid has been mentioned by a number of previous workers. Very little was known of the nature and properties of these reductants and they were generally assumed not to affect the vitamin C value of foods as determined by dye titrations, either because they were not present in significant amounts or because they reacted more slowly with the dye so that their interference was obviated by carrying out the titration very rapidly, the end-point being reached in a

minute or less. However, some doubt has been cast on this assumption by the recent findings of Hochberg, Melnick & Oser [1943] and Taylor [1943] that, when the end-point is reached still more rapidly by extrapolation of photoelectric data, the results may be appreciably lower than those given by the ordinary visual methods. Taylor [1943] found the total vitamin C in unripe walnuts to be about 13% less by the more rapid method, and similar discrepancies occurred with other foods.

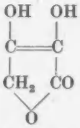
Wokes *et al.* [1943*b*], using a potentiometric method, have shown that the vitamin C value obtained for samples of dehydrated carrots, in which much apparent vitamin C had developed during 4 years' storage, was increased as longer intervals were allowed between the dye additions, thus allowing more time for the apparent vitamin C to react. With additions every 15 sec. the result was about 40 mg./100 g.; with additions every 8 min. the result was over 100 mg./100 g. It was suggested that this was due in part to enol-keto tautomerism. Apparent vitamin C was thought to exist in the solutions undergoing titration as a mixture of two isomers. Only one of these reacts with the dye. As the dye is added it removes the active isomer, and more of this is formed from the inactive isomer to restore the equilibrium. Thus the time effect is due to the rate of enol-keto tautomerism as well as to the rate of reaction of the active form with the dye.

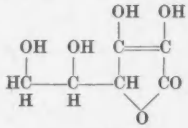
In the present investigation it has been found that apparent vitamin C can be formed under much less drastic conditions, and occurs more widely and in greater proportions than was previously supposed. Moreover, its components resemble true vitamin C closely (1) in their susceptibility to oxidation by very low concentrations of inorganic or organic peroxides—an oxidation which is accelerated by enzyme extracts containing ascorbic acid oxidase, (2) in their removal by activated charcoal and general behaviour towards adsorbents, and (3) in their reduction of iodine, ammoniacal silver nitrate or alkaline copper solutions (Benedict's, Fehling's) in the cold. Some of the reductants in apparent vitamin C are gradually destroyed on standing in HPO_3 solution at room temperature, but may be stabilized by nitrogen or cyanide; others are stable in HPO_3 solution. They also differ from vitamin C in possessing no antiscorbutic activity and in being more stable towards heat and towards formaldehyde at pH 4–5 [Wokes *et al.* 1943*b*]. They should resemble ascorbic acid closely in chemical constitution and contain the active dienol and carbonyl groups. These are present in reductone, reductic acid, dihydroxymaleic acid and hydroxytetronic acid, four compounds closely related to ascorbic acid, as shown by comparison of their formulae.


Reductone

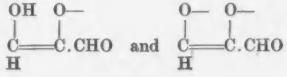

Dihydroxymaleic acid

Reductic acid


Hydroxytetronic acid


Ascorbic acid

Reductone is formed, amongst other products, when carbohydrates are treated with alkali. Such treatment may produce a variety of enolic compounds, hence the 'reductones' which Mapson prepared may have contained other dye reductants besides pure reductone, especially as their properties differed in certain respects (e.g. stability towards HPO_3) from those of our apparent vitamin C. Martius & Euler [1934] showed that reductone and dihydroxymaleic acid react much more slowly with the dye when the pH is >5, and suggested that reductone could dissociate into two tautomeric ionized forms



Snow & Zilva [1938], when describing their experiments with ascorbic acid oxidase on reductone, suggested that their findings might be explained by the high dye-reducing power of one of these isomers, the concentration of which would be controlled by the velocity of the tautomeric conversion. A similar explanation is suggested for the variations we have observed in the reaction velocity of the dye with apparent vitamin C in different foodstuffs. Reductone has an absorption maximum at 287mμ above pH 5·7 and at 268mμ at pH 4·0 or lower, indicating a change in the proportion of the two isomers in this pH range which may be related to the difference in properties of apparent vitamin C in foods of pH above or below this range. The maximum at 268mμ would be in good agreement with the maximum of 264mμ found in our beet molasses at a similar pH, but the evidence for the presence of reductone is not conclusive. Euler & Martius [1933] stated that reductone has no antiscorbutic activity.

Reductic acid was obtained by Reichstein & Oppenauer [1933] by heating beet sugar pectin or various carbohydrate derivatives with mineral acid, treatment like that which produced apparent vitamin C in our molasses. Reductic acid has an ab-

sorption maximum at 280mμ at pH 6 and at 264mμ at pH 3 [Mayer, 1937]. The spectroscopic data on our materials do not preclude the presence of reductic acid, but the fact that the apparent vitamin C was not completely destroyed on standing in HPO_3 [Wokes *et al.* 1943*b*] suggested that the latter contained at least two different substances.

Dihydroxymaleic acid is formed when tartaric acid is oxidized by H_2O_2 in presence of small quantities of ferrous salts in sunlight. Szent-Györgyi [1939] states that it is quite common in plants, where it probably serves as a hydrogen transmitter analogous to ascorbic acid, being reversibly oxidized by an oxidase similar to ascorbic acid oxidase. Dihydroxymaleic acid, like ascorbic acid, is rapidly oxidized by traces of peroxides, but unlike ascorbic acid is unstable in acid solutions, in which it resembles part of the apparent vitamin C in our materials. Solutions of dihydroxymaleic acid in HPO_3 are not stabilized by nitrogen or cyanide. Such stabilization might be effected in the presence of certain food extracts, but our experiments in this direction have so far proved unsuccessful. Potentiometric studies of its rate of reaction with the dye confirm Szent-Györgyi's suggestion that in aqueous solution it is subject to enol-keto tautomerism. The maximum light-absorption for dihydroxymaleic acid in water is at 290mμ. This differs appreciably from the maximum at 280mμ in our incubated malt extract, and the maximum at 275–276mμ in the molasses, but the possibility must be considered that the acid in the molasses may have shifted the dihydroxymaleic acid maximum from 290 mμ to a lower wave-length. The presence of dihydroxymaleic acid in our materials is therefore not excluded. The destruction of the non-specific reductant in these materials by our enzyme extracts may have been due to their containing dihydroxymaleic acid oxidase, which Banga & Philipot [1938] found in many plants. Dihydroxymaleic acid is not antiscorbutic [Dalmer, 1934].

Hydroxytetronic acid is chemically of interest as being the parent substance from which ascorbic acid is probably formed. It resembles ascorbic acid closely in its rate of reaction with HCHO and with the indophenol dye. Its maximum absorption at 246mμ is appreciably lower than the nearest maximum of 256mμ in any of our materials, and would presumably tend to become still lower in presence of acid. Its occurrence in our materials seems less likely than that of reductic or dihydroxymaleic acids. Hydroxytetronic acid is not antiscorbutic [Micheel & Jung, 1934].

Other possible constituents of apparent vitamin C. It appears from spectroscopic evidence, and from the effects of HPO_3 and HCHO at different pH [Wokes *et al.* 1943*b*], that apparent vitamin C frequently contains more than one non-specific reductant. In addition to the reductants mentioned above, enolic substances may be present which reduce the dye fairly rapidly but which are less closely related to ascorbic acid. Certain German workers [cf. Enders, 1938; Luers, 1937] have described the occurrence and gradual development, in malted barley extracts and beer during storage, of dye reductants which they state are formed by condensation of sugars and amino-acids with evolution of CO_2. These reductants are stated not to reduce Fehling's solution, which indicates that their chemical nature differs more widely from that of ascorbic acid than does that of the above four reductants.

SUMMARY

1. The provisional term 'apparent vitamin C' is proposed to describe a group of substances occurring in foods and closely resembling ascorbic acid in chemical and physical properties (as well as in behaviour towards oxidizing enzymes), so that they are not distinguished from vitamin C by the dye titration generally employed.

2. These substances probably all contain the dienol group, and may include reductone, reductic acid, dihydroxymaleic acid and hydroxytetronic acid. They undergo enol-keto tautomerism, which causes their rate of reaction with the dye, and other properties, to be affected by the pH, the range 4–6 appearing to be critical.

3. They differ from ascorbic acid in being produced by the action of heat on certain constituents (e.g. pectins, carbohydrates) in foods and also (except hydroxytetronic acid) in their rate of reaction with formaldehyde under given conditions [Wokes *et al.* 1943*b*]. These differences in behaviour have formed the basis of methods of estimating apparent vitamin C in foodstuffs.

4. Apparent vitamin C has been found in germinated grains, malt extract, cocoa, chocolate, dehydrated fruits and vegetables, beer, and in certain fruits and herbs such as walnuts, parsley and sorrel. Particularly high contents have been found in unripe walnuts, in concentrated preparations from beet and cane molasses, and in fruit juices and dried foods stored for several years under normal conditions.

The apparent vitamin C content of processed and dehydrated foods may gradually increase during storage under normal conditions, and thus obscure loss of true vitamin C in storage experiments.

We are indebted to Dr L. J. Harris and his colleagues for advice and criticism, to Dr R. A. Morton for spectroscopic examination of various samples, to Mr H. C. Powers, chief chemist, Messrs Tate and Lyle's Thames Refinery, for samples of molasses, to Messrs W. J. Bush and Co. for samples of fruit juices and syrups, and to Dr F. Bergel for specimens of reductic and hydroxytetronic acid.

REFERENCES

Ahmad, B. [1935]. *Biochem. J.* **29**, 275.
Bacharach, A. L., Cook, P. M. & Smith, E. L. [1934]. *Biochem. J.* **28**, 1038.
Banga, I. & Philipot, E. [1938]. *Hoppe-Seyl. Z.* **258**, 147.
Dalmer, O. [1934]. *Dtsch. med. Wschr.* **60**, 1200.
Enders, C. [1938]. *Kolloidzschr.* **85**, 74.
Euler, H. v. & Martius, C. [1933]. *Liebigs Ann.* **505**, 73.
Griffiths, J. G. A. [1943]. *Nature, Lond.*, **152**, 163.
Harris, L. J. [1933]. *Nature, Lond.*, **132**, 27.
—— Mapson, L. W. & Wang, Y. L. [1942]. *Biochem. J.* **36**, 183.
—— & Olliver, M. [1942]. *Biochem. J.* **36**, 155.
Hochberg, M., Melnick, D. & Oser, B. L. [1943]. *Industr. Engng Chem.* (Anal. ed.), **15**, 182.
Hopkins, F. G. & Morgan, E. J. [1936]. *Biochem. J.* **30**, 1446.
Johnson, S. W. [1933]. *Biochem. J.* **27**, 1942.
Levy, L. F. & Fox, F. W. [1935]. *S. Afr. med. J.* **9**, 181.
Luers, H. [1937]. *Münch. brautech. Zbl.* **36**, 11.
Lugg, J. W. H. [1942]. *Aust. J. exp. Biol. med. Sci.* **20**, 273.
Mapson, L. W. [1943*a*]. *Nature, Lond.*, **152**, 13.
—— [1943*b*]. *J. Soc. chem. Ind., Lond.* **62**, 223.
Martius, C. & Euler, H. v. [1934]. *Biochem. Z.* **271**, 9.
Mayer, N. [1937]. *J. Chim. phys.* **34**, 109.
McHenry, E. W. & Graham, M. [1935]. *Biochem. J.* **29**, 2013.
Melville, R., Wokes, F. & Organ, J. G. [1943]. *Nature, Lond.*, **152**, 447.
Micheel, F. & Jung, F. [1934]. *Ber. dtsch. chem. Ges.* **67** B, 1660.
Mirimanoff, A. & Mori, M. [1940]. *Schweiz. Apoth Ztg*, **78**, 685.
Reedman, E. J. & McHenry, E. W. [1938]. *Biochem. J.* **32**, 85.
Reichstein, T. & Oppenauer, R. [1933]. *Helv. chim. Acta*, **16**, 988.
Snow, G. A. & Zilva, S. S. [1938]. *Biochem. J.* **32**, 1926.
Szent-Györgyi, A. V. [1931]. *J. biol. Chem.* **90**, 384.
—— [1939]. *Oxidation, Fermentation, Vitamins, Health and Disease.* Baltimore: Williams and Wilkins.
Taylor, A. McM. [1943]. *Biochem. J.* **37**, 54.
Wokes, F. [1943]. *Nature, Lond.*, **152**, 328.
—— Johnson, E. H., Duncan, J., Organ, J. G. & Jacoby, F. C. [1942]. *Quart. J. Pharm.* **15**, 314.
—— & Organ, J. G. [1943]. *Biochem. J.* **37**, 259.
—— —— Duncan, J. & Jacoby, F. C. [1943*a*]. *Nature, Lond.*, **152**, 14.
—— —— & Jacoby, F. C. [1943*b*]. *J. Soc. chem. Ind., Lond.* **62**, 232.

The Osmotic Pressure of 'Defatted' Human Serum

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McCarthy showed [1938] that foetal sheep serum exerts a significantly higher osmotic pressure per gram of protein than does the maternal serum. This phenomenon could not be explained by the different albumin-globulin ratios of the two sera. Molecular weight determinations by McCarthy [1942] did not reveal any striking difference between foetal and maternal crystalline horse-serum albumins. Assuming that maternal and foetal serum albumins in the sheep are also identical, as is probable in the horse, we sought another explanation for the phenomenon observed in the foetal sheep serum. Since the lipid content of foetal blood is considerably less than that of the maternal blood [data for human: Slemons & Stander, 1923], and since Macheboeuf [1929], Macheboeuf & Sandor [1931] have stated that the lipid constituents of serum protein exert a considerable influence on osmotic pressure, it seemed desirable to investigate further the effect of lipids on the osmotic pressure of the serum proteins. Experiments were therefore undertaken to compare the osmotic pressure of serum before and after lipid extraction. The chief problem regarding the extraction was to find a suitable method which would remove the lipids without denaturing the serum proteins.

The method of McFarlane [1942] seemed the most convenient for our purpose, the method being also of especial interest since it is used in preparing pooled sera for transfusion. The method consists of shaking the serum with ether and freezing the mixture by cooling it to below $-25°$, then thawing it. The ethereal layer which separates during thawing contains a large proportion of serum lipids, and by repeated extractions approximately three-quarters of the lipids can be removed. McFarlane showed by electrophoretic measurements that the only change that takes place in the serum proteins is the diminution of the β-globulins.

METHODS

We extracted fresh pooled human serum by McFarlane's method. The serum was shaken with ether, frozen and thawed five times. Since the method employed by us may

differ slightly in detail from that of McFarlane, a summary of our procedure is desirable. The serum was frozen by surrounding the container with solid carbon dioxide ('Dricold', Imperial Chemical Industries Ltd.) and leaving it for several hours or overnight. The frozen serum was thawed at room temperature by inverting the vessel and letting the thawed liquid drip into another container. It was noticed that as the thawed serum collected, several layers separated out: usually the bottom part was crystal clear and the rest increasingly milky towards the top. After the yellow ethereal layer had been siphoned off and the extracted serum stirred up, the serum lost its opacity and became crystal clear throughout after each extraction, so that there was no need to centrifuge or filter it. While the frozen serum was thawing, a thick, creamy, yellowish material gradually separated on the surface of the ice. This remained in the first vessel; it had a low solubility in water and was not further analyzed. This material may be identical with the gelatinous deposit of low solubility in water, which McFarlane found to separate, after freezing and thawing the serum, subsequently to the last ether extraction, and which had to be filtered or centrifuged off to clear the serum. After the last ether extract had been siphoned off, the ether remaining dissolved in the serum was removed by passing nitrogen gas through the serum for approximately 1 hr. at 0°. After this, samples of the extracted serum and of the untreated serum, the latter having been kept frozen up to this time, were placed in collodion membranes and dialyzed for 48 hr. at 0° against M/15 phosphate buffer (M/30 $Na_2HPO_4.12H_2O$ + M/30 KH_2PO_4) of pH 6·8. Ten osmometers were set up at 0° with various dilutions of each sample of serum according to the method of Adair [1925], with the same buffer for the outer fluid as in dialysis. The protein concentration of the serum in each osmometer was measured by a micro-Kjeldahl determination. Albumin N was determined on three of the more concentrated sera after precipitation of the globulins by saturation with $MgSO_4$.

In calculation of protein concentration from N content, since the N contents of human-serum albumin and globulin are not known, the figures for horse-serum albumin (15·6% N) and horse-serum globulin (15·13% N) [Adair & Robinson, 1930*a*] were used. No allowance has been made for the presence of phospholipid N in the normal serum, nor for the loss of this after the extraction, since the corrections that might have been introduced are insignificant in comparison with the probable error in the determinations of total N. The serum was analyzed for its lipid content before and after the extraction. The distribution of lipids in the ether extract, and the N content of the latter, were also determined. Total fatty acids, fatty acids derived from neutral fats and cholesteryl esters, and total phospholipids were all determined by the methods of Bloor [1928, 1929]. Total free and ester cholesterol were estimated by the digitonin method as described by Popják [1943].

RESULTS

Table 1 shows the lipid content of the serum before and after extraction. It can be seen that practically all of the cholesterol, approximately two-thirds of the phospholipids and two-thirds of the neutral fats have been removed by the extraction. Table 2 shows the observed percentage distribution of lipids in the dry ether extract, together with the theoretical distribution calculated from the results of lipid analyses of the sera, assuming that all the lipids removed by the extraction were to be recovered in the ether extract. It is evident that the observed and theoretical lipid distributions differ considerably; the results appear to indicate that a large proportion of the phospholipids, neutral fats, and also of free cholesterol was lost with the material which separated on the surface of the ice during each thawing.

Table 1. *Lipid content of serum before and after extraction*

	Before extraction (mg./100 ml.)	After extraction (mg./100 ml.)
Cholesterol: Free	44	—
Ester	148	—
Total	192	Traces
Total fatty acids	390	101
Fatty acids*	280	61
Neutral fats†	183	64
Total phospholipids	171	61
Total lipids	652	125

* Fatty acids derived from neutral fats and cholesteryl esters.

† Neutral fats = (fatty acids − ester cholesterol × 0·72) × 1·05.

Table 2. *Observed and theoretical percentage distribution of lipids in ether extract of serum*

	Lipid content of ether extract	
	Observed (%)	Theoretical (%)
Phospholipid	3·20	20·90
Neutral fat	4·20	22·60
Cholesterol: Free	2·00	8·35
Ester	56·00	28·05
Fatty acids of cholesteryl esters	36·00	20·10
Total	101·40	100·00

The N content of the ether extract was found to be 0·28 mg./100 mg. of dry extract; about 22% of this small amount of N is accounted for by the phospholipids. If the remaining fraction of N is protein-N, the amount of protein in the extract must have been negligibly small.

Total N determinations on the dialyzed normal and 'defatted' sera showed a discrepancy in total protein concentration of a magnitude difficult to reconcile with any observed volume changes due to dilution in the dialysis. The total protein concentration of the dialyzed normal serum was 6·82% and that of the dialyzed 'defatted' serum 5·49% (cf. Table 3, osmometers 1 and 11 respectively). It seems probable that N determination and lipid analysis on the material which separated out on the surface of the ice during thawing would have explained the discrepancies.

The percentage albumin and globulin contents of the dialyzed normal serum were 56·76 and 43·24% respectively; and those of the dialyzed extracted serum 61·64 and 38·36% respectively, indicating a diminution of globulin content. It is of interest to note that a loss of β-globulin following the ether extraction of the serum was reported by McFarlane on the basis of electrophoretic analysis, although no quantitative data are available in his paper.

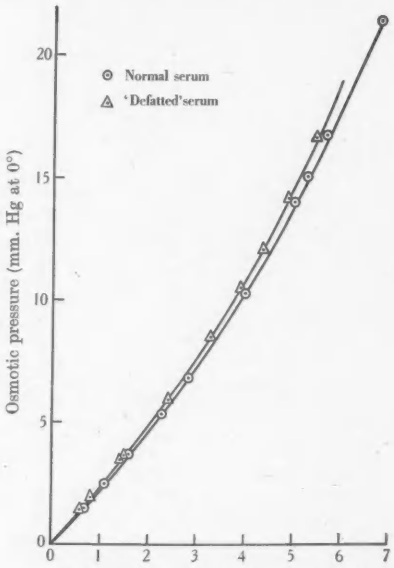

Fig. 1. Relationship between protein concentration and osmotic pressure of normal and of 'defatted' serum. The data for the two curves have been calculated from the equation of Adair: $p = \pi_0 C/(1 - K_b C)$ [cf. Adair & Robinson, 1930*b*]. K_b (normal serum) = 0·0509; K_b' (defatted serum) = 0·0547. The circles and triangles represent observed values.

Fig. 1 illustrates the osmotic pressures, in mm. of Hg at 0°, of the normal and of the extracted sera; the detailed data are presented in Table 3. It would appear that the 'defatted' serum exerts slightly higher osmotic pressures for the same protein concentrations than the normal serum. The difference in higher concentrations amounts to 0·5–0·6 mm. of Hg, in lower concentrations to 0·2–0·3 mm. of Hg. Evidence on the question whether this difference is due to the removal of lipids, or to the higher percentage of albumin in the extracted serum, can be obtained by finding the value of π_0 for both sera, i.e. the limiting value of π, i.e. the ratio

$$\frac{p \text{ (osmotic pressure)}}{C \text{ (protein concentration)}}$$

Table 3. *Data for osmotic pressures of normal and 'defatted' sera*

Osmometer no.	Osmotic pressure (mm. Hg at 0°)	Protein concentration (g./100 ml. solution)	$1/\pi$
	(*a*) Normal serum		
1	21·39	6·82	0·319
2	16·77	5·69	0·339
3	15·05	5·28	0·351
4	13·97	5·01	0·359
5	10·22	3·99	0·391
6	6·77	2·82	0·417
7	5·30	2·28	0·431
8	3·64	1·57	0·431
9	2·48	1·11	0·448
10	1·44	0·68	0·472
	(*b*) 'Defatted' serum		
11	16·66	5·49	0·329
12	14·20	4·91	0·346
13	12·11	4·35	0·360
14	10·49	3·91	0·373
15	8·50	3·28	0·386
16	5·86	2·41	0·411
17	3·63	1·51	0·417
18	3·47	1·45	0·418
19	1·95	0·82	0·420
20	1·39	0·62	0·448

in an infinitely dilute solution, and then comparing the observed and theoretical values of π_0. In the

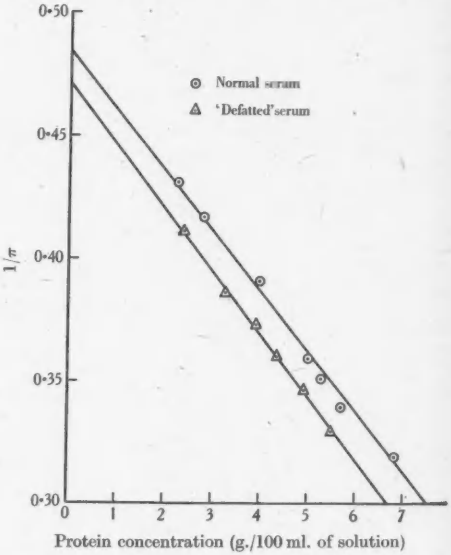

Fig. 2. Relationship between protein concentration and $1/\pi$ ($= C/p$).

comparison of osmotic pressures, it should be noted that the unequal distribution of ions may affect

results for the more concentrated solutions. This complication is eliminated by calculation of the value of π_0 for infinite dilution [Adair & Robinson, 1930*b*]. In Fig. 2 observed values of $1/\pi$ are plotted against protein concentration. The straight lines have been drawn visually as the best fit for values of $1/\pi$ relating to protein concentrations above 2 g./100 ml. of solution, and the values of $1/\pi_0$ determined by extrapolation of these lines to the ordinate. The points relating to lower protein concentrations are neglected, as the unavoidable experimental errors at these concentrations produce significant deviations in the value of $1/\pi$. The values of $1/\pi_0$ for the normal and extracted sera thus determined by extrapolation are 0·4835 and 0·4720 respectively [cf. Adair & Robinson, 1930*b*], and hence π_0 for the normal serum is 2·07, and for the extracted serum 2·12. Adair & Robinson [1930*b*] showed that for an infinitely dilute solution of serum the osmotic pressure can be expressed by the formula

$$p = RT\,[10C_a]/M_a + RT\,[10C_g]/M_g,$$

where C_a and C_g denote the concentrations (g./100 ml. of solution) of albumin and globulin, and M_a and M_g the molecular weights of albumin and globulin. In calculating the theoretical π_0, it is assumed that $M_a = 72{,}000$ and $M_g = 170{,}000$, values known for horse-serum albumin and globulin [Adair & Robinson, 1930*b*]. Then the value of theoretical π_0 is calculated by the formula

$$\pi_0 = 2{\cdot}36X_a + 1{\cdot}00\,(1 - X_a),$$

where X_a = g. of albumin/g. of total protein. For the normal serum $X_a = 0{\cdot}568$ and for the extracted serum $X_a = 0{\cdot}616$. Therefore the theoretical values of π_0 for the normal and extracted sera are 1·77 and 1·84 respectively. Thus there is an increase in the value of the theoretical π_0 of the same order as in the observed values, which is attributable to the increased percentage of albumin in the extracted serum. The difference between the values of determined and theoretical π_0 for human serum proteins may be explained by the fact that constants for horse-serum proteins were used in the computation of theoretical π_0.

Therefore we conclude from our experiment (*a*) that McFarlane's method for extracting lipids from sera removes also a part of the globulin complex without altering the colloid osmotic pressure properties of the remaining serum proteins, and (*b*) that the lipids extracted by the method do not influence the osmotic pressure of the serum proteins. The higher osmotic pressure effect of foetal serum remains still unexplained.

SUMMARY

1. Fresh pooled human serum was 'defatted' by McFarlane's method.

2. Lipid analysis of serum before and after extraction showed that practically all of the cholesterol, approximately two-thirds of the phospholipids, and two-thirds of the neutral fats, were removed by the extraction.

3. The extracted serum exerts slightly higher osmotic pressure per g. of protein than the normal serum. This difference, however, can be explained by the increased albumin-globulin ratio in the extracted serum.

4. The serum lipids, which can be removed by McFarlane's method, do not influence the osmotic pressure of the serum proteins.

We are ind ebted to Mr G. S. Adair, F.R.S., for his helpful criticism of our results and to Prof. F. G. Young for kindly allowing us to use the cold room of his department. One of us (E. F. M.) wishes to express his indebtedness to Prof. W. G. Barnard for hospitality and facilities in his department.

REFERENCES

Adair, G. S. [1925]. *Proc. roy. Soc.* A, **108**, 627.
—— & Robinson, M. E. [1930*a*]. *Biochem. J.* **24**, 993.
—— —— [1930*b*]. *Biochem. J.* **24**, 1864.
Bloor, W. R. [1928]. *J. biol. Chem.* **77**, 53.
—— [1929]. *J. biol. Chem.* **82**, 273.
McCarthy, E. F. [1938]. *J. Physiol.* **93**, 81.
—— [1942]. *J. Physiol.* **101**, 314.
McFarlane, A. S. [1942]. *Nature, Lond.*, **149**, 439.
Macheboeuf, M. A. [1929]. *Bull. Soc. Chim. biol.* **11**, 485.
—— & Sandor, G. [1931]. *Bull. Soc. Chim. biol.* **13**, 745.
Popják, G. [1943]. *Biochem. J.* **37**, 468.
Slemons, J. M. & Stander, H. J. [1923]. *Bull. Johns Hopkins Hosp.* **34**, 7.

The Estimation of 'True Ascorbic Acid' in Blood

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Recent work has suggested that the blood ascorbic acid concentration may provide a satisfactory index of the nutritional level of vitamin C. The concentration in whole blood is preferable for this purpose to the plasma concentration, which shows greater fluctuation; but the chief disadvantage of the blood ascorbic acid concentration as a nutritional index is the technical difficulty of estimating it accurately. The normal values obtained by different sets of workers show wide variation, and there is considerable doubt as to how much of the reducing substances estimated by these methods represents true ascorbic acid. For this reason Butler & Cushman [1940] suggest the use of the term 'apparent ascorbic acid' for the blood estimates, since they conclude that 'satisfactory criteria for the validity of whole blood analyses are lacking'.

In the present investigation the simple indophenol titration method of Deeny, Murdock & Rogan [1942] was chosen for special study. Preliminary experiments showed that a significant quantity of non-ascorbic acid reducing substance could be removed by treating the blood filtrates with lead acetate and so obtaining a precipitate of lead phosphate. This procedure removes certain SH derivatives, but not ascorbic acid [Richter & Croft, 1943]. Blood ascorbic acid figures obtained by this lead precipitation method were then compared with figures obtained on the same samples by the ascorbic oxidase method. Good agreement between estimates obtained by these two distinct methods is considered to be satisfactory evidence of the validity of the blood ascorbic acid determination.

The lead precipitation method described is very simple, and as it requires no special apparatus it appears very suitable for use in clinical investigation.

METHODS

Venous blood containing sodium oxalate (0·30 g./100 ml.) or sodium citrate (0·05 ml. saturated solution to 5 ml. blood) was used. Special care was taken to avoid haemolysis.

Lead precipitation procedure. The oxalated blood (4 ml.) was put in a 15 ml. centrifuge tube containing 0·05 ml. *sec.*-octanol, and coal gas was bubbled through. Distilled water (4 ml.) was added and the gassing continued for 10 min. 2 ml. of freshly prepared 32 % HPO_3 were added, followed after mixing by 1 ml. 25 % $Pb(Ac)_2.3H_2O$ and 2 ml. saturated sodium acetate solution. After thorough mixing, the suspension was centrifuged. Any cloudiness in the supernatant fluid indicated that the HPO_3 solution was not sufficiently fresh. 2 ml. portions of the supernatant solution were taken for titration.

The dye solution (2 mg. 2:6-dichlorophenolindophenol in 100 ml. warm water) was standardized by titration against a freshly made solution containing 2 mg. ascorbic acid in 400 ml. 2 % HPO_3. On one occasion it was found that erroneous results were due to a rapid fading of the dye solution (30 % in 24 hr.). This was traced to an impurity in the particular specimen of dye. Other specimens supplied by the same firm gave satisfactorily stable solutions, but the stability of the dye solutions should be checked before use.

The dye solution was added from a 2 ml. microburette to 2·0 ml. ascorbic acid solution in a small conical vessel made from the end of a conical centrifuge tube. The solution was stirred with a glass rod after each addition and the titration was continued until a faint pink colour persisted for 10 sec. and matched a solution made by adding 0·05 ml. of dye to 1 ml. of 1 % HPO_3 in a similar conical vessel: the amount of dye solution added in the blank was subtracted from the final burette reading. As the pink colour at the end-point was very faint, it was essential that the titration should be done against a white background and in a good light. After some practice, duplicate estimations showed good agreement.

In calculation of the result a small correction was applied for the ascorbic acid contained in the protein precipitate obtained in deproteinization. The precipitate was found to contain 65 % of water, by drying to constant weight. With the volumes of blood and reagents given the total volume was 13 ml., of which about 2·2 ml. were occupied by the moist precipitate. The effective volume of the aqueous solution was therefore taken as 12·2 ml. The ascorbic acid concentration in whole blood (mg./100 ml.) was obtained by multiplying the mg. ascorbic acid found in 2 ml. of the supernatant solution by the factor $\frac{1}{2}(12{\cdot}2) \times \frac{1}{4}(100) = 152{\cdot}5$.

RESULTS

Removal of non-ascorbic acid reducing substances with lead acetate. Ascorbic acid estimations were carried out on a series of blood specimens by the method of Deeny *et al.* [1942]. It was then found that when the blood filtrates obtained after deproteinization with HPO_3 and sodium acetate were treated with 1 ml. 25% lead acetate solution, and the lead phosphate precipitate was removed by centrifuging, titration with the indophenol dye generally gave a significantly lower result, owing to the removal of non-ascorbic acid reducing substances (Table 1). Allowance has been made for the dilution by the lead acetate solution.

Table 1. *Ascorbic acid content of fresh and of stored blood*

Time of storage (days)	Total dye reducing substance, estimated as ascorbic acid (mg./100 ml.)	'True ascorbic acid' by lead precipitation (mg./100 ml.)	Non-ascorbic acid reducing substance (%)
	(1) Oxalated blood		
0	0·78	0·70	11
0	0·60	0·54	11
	(2) Citrated blood		
0	0·58	0·54	7
2	0·57	0·43	33
6	0·63	0·57	10
6	0·46	0·38	21
6	0·46	0·38	21
6	0·25	0·23	9
6	0·67	0·50	34

The stored specimens were kept at 3°.

Estimation by ascorbic acid oxidase. That the lower ascorbic acid figures obtained after lead acetate treatment were due to the removal of non-ascorbic acid reducing substances was confirmed by estimating the ascorbic acid by an independent method. The ascorbic oxidase was used for the quantitative oxidation of the ascorbic acid. The oxidase was prepared from cauliflower and the experimental procedure was that of Richter & Croft [1943]. The use of this method was made difficult by the relatively low concentration of ascorbic acid in the blood filtrates and for this reason the method cannot be recommended for blood; but with great care satisfactory agreement of duplicates was obtained. Each figure given in Table 2 represents the means of two separate estimations, i.e. of six burette readings.

It was clear that the figures obtained by the ascorbic oxidase method agreed very much more closely with those obtained by the lead precipitation method procedure (correlation coefficient 1·00) than with the figures for the total reducing substance given by the method of Deeny *et al.* [1942] (correlation coefficient 0·96). The closeness of the agreement gave evidence of the reliability of the lead precipitation method.

Table 2. *Estimation of ascorbic acid in blood by (a) ascorbic acid oxidase and by (b) lead methods*

Total reducing substance as ascorbic acid (mg./100 ml.)	'True ascorbic acid' (a) By oxidase method (mg./100 ml.)	'True ascorbic acid' (b) By lead method (mg./100 ml.)
	(1) Oxalated blood	
0·50	0·37	0·36
0·36	0·31	0·30
0·22	0·21	0·21
0·62	0·56	0·56
	(2) Citrated blood	
0·51	0·42	0·44
0·31	0·27	0·26
0·40	0·30	0·32

The correlation coefficient between the total reducing substance and the figures obtained by the ascorbic oxidase method was 0·96: between figures by the lead precipitation method and the ascorbic oxidase method, 1·00. The blood specimens were used within 3 hr. of collection.

Subsequent work showed that it was unnecessary to carry out the two precipitations separately and to precipitate with lead acetate after a preliminary deproteinization: identical figures were obtained when these two stages were combined as described under 'Methods'.

The stability of ascorbic acid in citrated whole blood

Heinemann [1938] and Kassan & Roe [1940] reported that the level of ascorbic acid in whole blood remained constant for 16–24 hr. at room temperature and for 52 hr. at 0°. As it was desired to analyze specimens sent by rail and by post, this point was further investigated. Estimations were made on specimens of citrated and oxalated blood immediately after collection and after storage for 24 and 48 hr. at various temperatures, with or without occasional shaking (Table 3).

There was no significant loss of ascorbic acid under these conditions for 24 hr., and even in 48 hr. the maximum loss observed in 10 specimens was only 22%. The range of blood ascorbic acid concentrations is wide—from about 1·5 mg./100 ml. in the fully saturated individual to below 0·15 mg./100 ml. in a person with scurvy. In clinical work only the approximate level is usually required in order to demonstrate or to exclude the existence of gross deficiency. A loss of even 22% would not render the result entirely useless for this purpose.

Table 3. *Stability of ascorbic acid in citrated and oxalated whole blood*

Anti-coagulant	Percentage of initial ascorbic acid remaining after (hr. of storage) 0	24	48	Initial ascorbic acid content (mg./100 ml.)	Temp. (°)
Saturated citrate	100	100	—	0·30	16
	100	96	—	0·27	16
	100	98	91	0·55	14
	100	100	84	0·48	14
3·8% citrate	100	100	80	0·27	21
	100	—	100	0·19	20
	100	—	90	0·15	20
	100	—	78	0·30	20
	100	—	84	0·26	20
	100	97	91	0·32	19
Oxalate	100	97	85	0·34	20
	100	100	89	0·30	20

Blood preserved with saturated citrate contained 0·05 ml. saturated sodium citrate solution to 5 ml. blood; with 3·8% citrate, 2·5 ml. 3·8% sodium citrate solution to 10 ml. blood; with oxalate, 0·03 g. sodium oxalate to 10 ml. blood.

DISCUSSION

In attempts to assess the nutritional level of vitamin C in a subject, either the saturation test [Harris, 1942] or the blood ascorbic acid concentration can give useful information. The saturation test is simple to carry out, but its usefulness is limited in that it requires the accurate collection of urine, dosage with ascorbic acid and repeated estimations for a period of several days. The blood ascorbic acid concentration, on the other hand, is so constant that a single estimation is usually adequate, and it has the great advantage that the estimation need not be carried out immediately after collection of the blood specimens. Ascorbic acid is stabilized by the reducing systems present in citrated or oxalated whole blood, so that there is no significant loss on storage for 24 hr. at ordinary temperature. Estimations can thus be carried out satisfactorily even on specimens sent by post. The chief disadvantage of the blood concentration as a nutritional index is the difficulty of estimating it accurately. There is, however, no standard accepted method for the estimation of blood ascorbic acid, and some of the methods in current use are subject to serious criticism [Butler & Cushman, 1940]. Several photoelectric absorptiometric and other methods have been described which have given good results in the hands of experienced research workers, but they are too difficult for routine use by the average clinical laboratory technician and we must agree with Bessey [1938] that their accuracy has generally been overrated.

It is evident from the present investigation that the blood ascorbic acid figures obtained by methods such as that of Deeny *et al.* [1942] are too high, owing to the inclusion of a variable amount of extraneous reducing substances. The lead precipitation method described corrects this source of error and gives somewhat lower figures. In order to obtain the greatest possible accuracy a relatively large volume of blood (4 ml.) was used and the volume of the other additions was kept small. After some practice in recognition of the end-point of the titration, duplicate estimations showed very close agreement, and the reliability of the method was confirmed by comparison with the ascorbic oxidase method for estimating 'true ascorbic acid'.

The method has been applied satisfactorily to estimation of the blood ascorbic acid in factory workers in the Birmingham area: the blood specimens were sent by rail and titrated within 24 hr. at Mill Hill. The blood ascorbic acid concentration of 350 normal individuals varied from 0·15 to 0·85 mg./100 ml., with a mean level of 0·25 during the months of May, June and July 1943. Fruit and vegetables were scarce in the Birmingham area during this period.

SUMMARY

1. Figures obtained for the blood ascorbic acid concentration by certain current methods are too high owing to the inclusion of a variable amount of extraneous reducing substance.

2. A simple lead precipitation method is described which eliminates this source of error.

3. The validity of the estimates by the lead precipitation method was demonstrated by comparison with the ascorbic oxidase method for 'true ascorbic acid'.

4. There is no significant loss of ascorbic acid in specimens of citrated whole blood kept for 24 hr. at 20°.

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REFERENCES

Bessey, O. A. [1938]. *J. Amer. med. Ass.* **111**, 1290.
Butler, A. M. & Cushman, M. [1940]. *J. clin. Invest.* **19**, 459.
Deeny, J., Murdock, E. T. & Rogan, J. J. [1942]. *Biochem. J.* **36**, 271.
Harris, L. J. [1942]. *Lancet*, **1**, 642.
Heinemann, M. [1938]. *J. clin. Invest.* **17**, 751.
Kassan, R. J. & Roe, J. H. [1940]. *J. biol. Chem.* **133**, 579.
Richter, D. & Croft, P. G. [1943]. *Lancet*, **1**, 802.

The Estimation of Inositol in Animal Tissues

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Inositol occurs in animal tissues in both free and combined forms. The occurrence of water-soluble combined inositol was first suggested by Rosenberger [1908*a*,*b*, 1910]. Comparing the inositol contents of fresh rabbit muscle and commercial beef muscle, he came to the conclusion that animal tissues contain 'inositogen'. This may be identical with the 'tyrophenosit' of Danileffski [1884], who reported, without giving data, a considerable increase in the inositol content of mice after 6 days' autolysis at 37° in the presence of $CHCl_3$. Needham [1923] found that the skeletal muscle of a freshly killed rabbit contained only one-half the amount of inositol found in that of a rabbit killed 2 days previous to the estimation. An increase in the free inositol content of the skeletal muscle of rats occurred after autolysis of the tissues [Needham, 1924]. Winter [1934], working on cardiac muscle of dogs, postulated the occurrence of three forms of inositol in animal tissues: 'free' inositol, determined after ½ hr. boiling with 10% KOH; 'combined' inositol, determined after 5 hr. boiling with 10% KOH, and 'newly formed' inositol, liberated after incubation at 37° in nitrogen. The cardiac muscle of the sheep, ox and pig contained no 'combined' inositol [Winter, 1940]. It is doubtful, however, whether much significance can be attached to any of Winter's results, owing to the unreliability of the chemical methods he employed. Woolley [1941*a*] obtained inositol after acid or alkaline hydrolysis of an ethanol-insoluble, H_2O-soluble, non-dialyzable substance isolated from liver. This was considered to be probably a phosphoric acid ester of inositol. Rapoport [1940] found that in bird and turtle erythrocytes a large proportion of the organic acid-soluble P is present in the form of phytic acid. Besides being present in animal tissues in water-soluble combined forms, inositol also occurs in brain and spinal cord bound in a phosphatide fraction. Folch & Woolley [1942] isolated inositol from an acid hydrolysate of brain cephalin, and found that it constituted up to 10% of this phosphatide fraction and amounted to as much as 0·4% of the weight of the brain.

It is evident that further work is required to elucidate the nature of the water-soluble combined forms of inositol in the animal body and the methods of breakdown and significance of both water-soluble and water-insoluble combined forms. Recent developments have, in fact, followed on the application of a microbiological technique [Williams, Stout, Mitchell & McMahan, 1941; Woolley, 1942]. The chemical methods at present available for the estimation of inositol in biological material [see Needham, 1923, 1926; Winter, 1934, 1940; Young, 1934*a*,*b*; and Gregory, 1935] are, however, tedious, lengthy and involve the use of relatively large amounts of tissues. The method to be described is much quicker than previous ones and possesses several other advantages, including improvements in the extraction of inositol from tissues, the removal of interfering substances by ion exchange materials and the estimation of inositol, without isolation, by oxidation with periodic acid.

METHODS

Principle of the method of estimation

Dried tissue is extracted with water and the fractions insoluble in 70% acetone and soluble in ether both removed from the aqueous extract. Glucose is then removed by yeast fermentation and both acidic and basic substances present in the extract removed by adsorption on a mixture of ion exchange adsorbents. The free inositol present in the extract is then quantitatively oxidized with HIO_4, the excess HIO_4 being estimated iodometrically. Suitable corrections are applied for any HIO_4 used in oxidizing the glycerol which may be present in the extract at this stage.

Water-soluble combined inositol is determined after acid hydrolysis of the aqueous extract.

Experimental

Preparation of tissue for extraction. As soon as possible after death, the tissue is removed from the animal, sectioned on a freezing microtome and the sections dried in thin layers in Petri dishes *in vacuo* in the cold room over silica gel. Drying is completed in an oven at 110°, after which the material is powdered and kept in the cold until analyzed. The water content of another portion of the same tissue is determined by drying to constant weight at 110°.

Extraction of inositol. An amount of dried powdered tissue containing approximately 0·5–1·0 mg. inositol (i.e. 1·0–2·0 g. dry skeletal muscle powder) is brought to the boil with 50 ml. distilled water, filtered under reduced pressure through a small plug of cotton-wool in a funnel, and the vessel and filter washed with two portions of 5 ml. of distilled water. This process is repeated twice, the three

extracts pooled and evaporated to about 50 ml., and acetone added to give a final concentration of 70%. The precipitate is filtered off and the acetone removed from the filtrate by distillation. The extract is evaporated down to about 25 ml. and extracted with ether. The ether extract is washed once with about 20 ml. distilled water, and the washings added to the aqueous residue, which is then concentrated to approximately 30 ml. If it is desired to estimate the 'combined' water-soluble inositol as well as the 'free' inositol the aqueous extract can be divided into two portions and one hydrolyzed as described below.

Removal of glucose. The only satisfactory method for the removal of glucose was found to be yeast fermentation, as used by Young [1934*a*]. Since some products of glucose fermentation have to be removed on the ion exchange adsorbents, the fermentation stage was carried out before the adsorption.

A washed yeast suspension is prepared by shaking one part by weight of pressed baker's yeast with five parts of distilled water, and centrifuging; this process is repeated four times and the suspension of washed yeast then made up to the original volume with distilled water. The suspension is prepared fresh each day and its potency tested by incubating 2 ml. with 25 ml. glucose solution (containing 5 mg. glucose) for 10 min. at 37°, and estimating the reducing power of the supernatant fluid, obtained by centrifuging, by Hagedorn & Jensen's ferricyanide method, a blank being set up at the same time with yeast suspension and water only.

The tissue extract is incubated at 37° with 2 ml. of the yeast suspension for 10 min. and then centrifuged. The supernatant liquid is siphoned into a boiling tube and the yeast washed twice by centrifuging with 10 ml. portions of distilled water, the washing being kept separate from the main supernatant fluid for the subsequent adsorption.

Preparation of ion exchange materials and the removal of interfering substances from tissue extracts. After treatment with yeast, the extract is purified by the use of ion exchange materials, with a carbonaceous zeolite for cation exchanges and 'M.P.D. Resin' as an acid adsorbent. Both these are obtainable from Permutit Company, Limited. A brief account of the use of these substances for the purification of muscle extracts has been published [Platt & Glock, 1942]. The removal of creatine and creatinine from muscle extracts by this method proved to be much more efficient than by the precipitation technique of West & Petersen [1932] with $HgSO_4$ and $BaCO_3$, which was employed by Young [1934*b*]. Treatment of the extract with a mixture of regenerated, washed and dried carbonaceous zeolite and 'M.P.D. Resin' is more efficient than successive treatments with the two materials separately. With this technique, however, the adsorbing materials cannot be regenerated.

250 g. or more of the carbonaceous zeolite are shaken gently at intervals for a period of 1 hr. with 2 vols. of 2N HCl, filtered on a Buchner funnel and washed on the funnel with a total volume of approximately 5 l. of distilled water. The final washings should be neutral to litmus. This regenerated, washed cation exchange material is dried at 100° and sieved so that the particles are of 20/40 mesh sieve size. The 'M.P.D. Resin' is treated in a similar way, but with 2N NH_4OH instead of HCl. At least 1 l. of hot distilled water should be used to wash this reagent, in order to remove excess *m*-phenylene diamine which is liable to be present and which, if not removed completely, gives a blank reading with HIO_4. These two regenerated, washed, dried and sieved materials are mixed in equal parts by weight; this preparation will be called the 'adsorbent'.

15 g. of adsorbent are introduced into a tube approximately 20 in. by $\frac{7}{16}$ in., narrowed at the bottom to approximately $\frac{3}{16}$ in., and fitted with a small piece of rubber tubing carrying a glass tip and provided with a screw clip. The material is kept in position in the tube with a loosely packed plug of glass-wool. Before being treated with the tissue extract, the adsorbent is washed with a total volume of approximately 100 ml. of distilled water. The final washings must give no blank when allowed to react with 5 ml. 0·01 M HIO_4 for 10 min. at room temperature. To prevent the formation of air bubbles in the column, which would impede the passage of liquid, the tube should contain distilled water into which the adsorbent is poured, the level of the liquid being kept above the top of the adsorbent. After the adsorbent has been washed sufficiently, the supernatant liquid from the yeast treatment is poured into the tube and allowed to drip through the column at a rate such that the whole of the liquid passes through in approximately 90 min. When the liquid has passed through completely, the first 10 ml. portion of the yeast washings is poured into the tube, and the rate of flow adjusted so that this liquid passes through in approximately 15 min. The tube containing the column of adsorbent is sucked dry on a filter pump, and then a second 10 ml. portion of yeast washings is allowed to drip through the column, which is then sucked dry. The column is washed with the help of suction with 15 successive 5 ml. portions of distilled water. This amount of washing is found to be necessary and sufficient to obtain quantitative recovery of 1 mg. of added inositol.

The assumption has been made that the only HIO_4-reacting substances which are not removed from yeast-fermented muscle extracts by the adsorbent mixture are inositol and glycerol.

Oxidation with HIO_4. At the suggestion of Prof. H. Raistrick, we investigated the possibility of estimating inositol by oxidation with HIO_4. Malaprade [1928 *a*, *b*, 1934] found that polyhydroxy alcohols were oxidized quantitatively by HIO_4 at room temperature. Fleury & Joly [1937 *a*, *b*] used periodate for the estimation of inositol both in pure solution and in the presence of glucose, but under the conditions used by them the oxidation, when allowed to go to completion, used up much more periodate than was to be expected from the equation

$$C_6H_6(OH)_6 + 6HIO_4 = 6HIO_3 + 6H.COOH,$$

and they came to the conclusion that secondary reactions occurred.

By allowing the reaction to go to completion at low temperatures (6–8°), we have found that the utilization of HIO_4 is quantitative. Formic acid, determined by titrating to pH 5·5 with 0·01 N NaOH, with a mixture of chlorophenol red and bromocresol green as indicator, has been produced to the extent of about 90% of the theoretical amount by the time the utilization of HIO_4 has reached the theoretical figure. Thereafter no more formic acid is formed. Some typical results for the reaction between HIO_4 and inositol in varying concentrations at 8 and 30° are given in Fig. 1.

Differential oxidation of inositol and of glycerol by HIO_4. After the removal of glucose from muscle extracts by fermentation with yeast, small amounts of glycerol remain. Some, no doubt, is extracted from the muscle and an

amount equivalent to approximately 4% of the glucose fermented is produced by fermentation. Attempts to remove glycerol by making use of its slight solubility in ether, its reported solubility in dry ethyl acetate, and its fermentation by *Bacillus proteus*, all failed. Glycerol, in concentrations of 0·2–0·9 mg./100 ml. solution, is, however, oxidized completely and quantitatively by 2 ml. 0·01 M HIO_4 at 8° in 90 min.

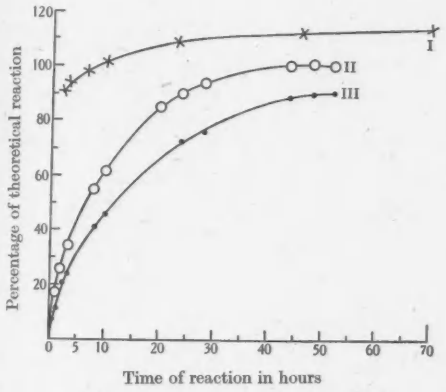

Fig. 1. Reaction of inositol with periodic acid. Extent to which the reaction

$$C_6H_6(OH)_6 + 6HIO_4 = 6H.COOH + 6HIO_3$$

is followed.

I, 1 mg. inositol/10 ml. + 5 ml. 0·01 M HIO_4 at 30°.
II, 1 mg. inositol/100 ml. + 10 ml. 0·01 M HIO_4 at 8°.
III, 1 mg. inositol/100 ml. + 5 ml. 0·01 M HIO_4 at 8°.

The percentage oxidation of glycerol (0·8 mg./100 ml.) by 2 ml. 0·01 M HIO_4 at 8° at intervals up to 100 min. is as follows:

Time in min.	20	30	40	60	70	80	90	100
% oxidation	53·0	68·6	79·8	95·6	98·0	100	100	100

The time for complete oxidation of glycerol depends on the temperature at which the reaction is carried out and on the concentration of both glycerol and HIO_4: e.g. at 6° 1 mg. of glycerol in 10 ml. water is completely oxidized by 3·0 ml. 0·01 M HIO_4 in 15 min.

Concentrations of inositol of 0·5–2·5 mg./100 ml. react to the extent of 1·7–2·2% with 2·0 ml. 0·01 M HIO_4 at 8° in 90 min. It was therefore necessary to determine (*a*) the total HIO_4-reacting material (inositol plus glycerol) in 100 ml. of solution treated with 10 ml. 0·01 M HIO_4 at 8° for 48 hr., and (*b*) the material reacting under the same conditions of concentration and temperature with 2 ml. 0·01 M HIO_4 in 90 min. (*b*) includes the whole of the glycerol and approximately 2% of the total inositol. Thus the difference between (*a*) and (*b*) multiplied by 1·02 gives the inositol content of the solution.

Absence of HIO_4-reducing substances other than glycerol and inositol from fermented effluent. There is support for the assumption, that glycerol and inositol are the only HIO_4-reacting substances left after fermentation and adsorption, in the fact that the glycerol content of the purified extracts, calculated from the HIO_4 reduced under the conditions stated above, is identical with that calculated from the formaldehyde formed, estimated as the dimedone derivative. The only other possible substances which would react with HIO_4 are amino-acids. Tests for NH_3 production (from β-hydroxy amino-acids) with HIO_4 in strongly alkaline solution in a Conway unit were always negative, as also were tests for amino-N by formol titration.

Estimation of free inositol. The effluent from the adsorption procedure is made up to 200 ml. in a graduated flask and 100 ml. withdrawn. Both portions are cooled to 8°. To one portion are added 10 ml. 0·01 M HIO_4 (also at 8°) and the reaction allowed to proceed for 48 hr. To the other portion, 2 ml. HIO_4 are added and the reaction is allowed to proceed at 8° for 90 min. If a temperature other than 8° is selected the time relationships for oxidation of both glycerol and inositol at this temperature must be determined. The reaction is stopped in both cases by adjusting the pH to approximately 6·5 by the addition of 5 ml. phosphate buffer (12 g. $Na_2HPO_4.12H_2O$ and 20 ml. N H_2SO_4/100 ml.), followed by 5 ml. 5% KI solution. After 5 min. the I_2 liberated is titrated with approximately 0·004 N $Na_2S_2O_3$ with 1% soluble starch in saturated NaCl as indicator, the end-point being stabilized by the addition of a few drops of saturated $NaHCO_3$ solution. 1 mg. inositol contained in 100 ml. distilled water is also allowed to react with 10 ml. 0·01 M HIO_4 for 48 hr. at 8°, and the excess HIO_4 titrated as above.

The HIO_4 reduced is obtained from the difference between the titration of the purified tissue extract after reaction with HIO_4 and the blank titration obtained with the HIO_4 alone.

Let x = HIO_4 reduced in terms of 0·004 N $Na_2S_2O_3$ in 48 hr. at 8°,

y = HIO_4 reduced in terms of 0·004 N $Na_2S_2O_3$ in 90 min. at 8°,

z = HIO_4 reduced by 1 mg. inositol in 48 hr. at 8°.

The approximate inositol content in mg. of half the extract is given by $(x-y)/z$ which should be multiplied by 1·02 (see above).

Estimation of 'combined inositol'. For the estimation of the water-soluble combined inositol, the extract is hydrolyzed with acid [Woolley, 1941*b*]. Both water-soluble fractions (i.e. 'free' and 'combined') are determined in the same sample of tissue, with twice as much tissue as in the procedure outlined above. Before fermentation, the extract is made to 50 ml., and one-half is refluxed for 6 hr. with HCl (final concn. 18% HCl). The HCl is removed by distillation under reduced pressure, and the contents of the flask are washed out with distilled water to give a total volume of approximately 25 ml. and then neutralized by the dropwise addition of N NaOH.

Experimental diets

Albino rats were put on an experimental diet, containing only small amounts of inositol, at 6 weeks of age and were in good condition when killed after an experimental period of 16½ months. The diet consisted of casein (A/E, Glaxo), 24%; sucrose, 67·5% (replaced by maize dextrin, after 13½ months on diet, for 2 months and by rice starch for last month); McCollum's salt mixture (185), 4%; wheat-germ oil, 2%; dried yeast (*Torula utilis*, 95% dry matter, containing not more than 2·7 mg. inositol/g.), 2·5%, in-

creased to 5% after 2 months on the diet; ascorbic acid 2·5 mg. daily; vitamin A approximately 22·5 I.U. daily and vitamin D approximately 4·5 I.U. daily (both contained in 2 drops of Radiostoleum diluted with peanut oil). Between 8 and 12·5 g. of this diet, mixed to a thick paste with water, were fed daily to each rat.

RESULTS

Inositol content of rat tissues

Table 1 contains the mean results of analyses of the tissues of four rats for water-soluble inositol, free and total (i.e. including combined).

Table 1. *Inositol content of rat tissues*

	Water-soluble inositol (mg./100 g. tissue)			
	Free		Total	
Tissue	Dry wt.	Wet wt.	Dry wt.	Wet wt.
(*a*) Rats given 5 mg. inositol/day				
Skeletal muscle	49·3	12·9	81·2	21·3
Liver	85·2	24·8	178	52·1
Heart	141·3	34·3	186	45·3
Brain	241	55·8	286	67·0
Kidney	328	88·0	456	123
(*b*) Rats given no extra inositol				
Skeletal muscle	24·0	6·3	76·2	18·3
Liver	89·0	25·5	174	50·3
Heart	—	—	—	—
Brain	169	37·8	295	66·1
Kidney	316	79·5	426	113

Our values for the total water-soluble inositol contents of rat tissues are of the same order as those found by other workers, including those recently obtained by Williams *et al.* [1941], with a microbiological method, on tissue autolysates. The proportions of free to total water-soluble inositol are also of the same order as those obtained by Woolley [1942], who also used a microbiological method.

It will be seen that there is no important difference between the values for the total water-soluble inositol for the rats receiving the inositol-deficient diet, and those with added inositol. It thus appears that rats are able to synthesize inositol, which confirms the earlier work of Needham [1924]. Woolley [1942] also showed that mice could synthesize inositol in the gut if the diet was not deficient in pantothenic acid.

SUMMARY

1. A chemical method is described for the estimation of inositol in animal tissues. This possesses advantages over earlier methods in that the time taken for an estimation is considerably reduced, the method of extraction of inositol from the tissues improved, the tedious precipitation procedures replaced by treatment with ion exchange materials, and the inositol finally estimated in solution by reaction with HIO_4 without isolation of the inositol.

2. Both free and total water-soluble inositol were determined in the kidney, heart, liver, brain and skeletal muscle of control rats, and of rats receiving a daily supplement of 5 mg. inositol. No appreciable difference was found between the total water-soluble inositol contents of the tissues in the two groups of rats.

REFERENCES

Danileffski, B. [1884]. *Bull. Soc. chim. Paris*, **41**, 255.
Fleury, P. & Joly, M. [1937*a*]. *J. pharm. chim.* **26**, 341.
—— —— [1937*b*]. *J. pharm. chim.* **26**, 397.
Folch, J. & Woolley, D. W. [1942]. *J. biol. Chem.* **142**, 963.
Gregory, R. A. [1935]. *Biochem. J.* **29**, 2798.
Malaprade, L. [1928*a*]. *C.R. Acad. Sci., Paris*, **186**, 238.
—— [1928*b*]. *Bull. Soc. chim. Fr.* (4th ser.), **43**, 683.
—— [1934]. *Bull. Soc. chim. Fr.* (5th ser.), **1**, 833.
Needham, J. [1923]. *Biochem. J.* **17**, 422.
—— [1924]. *Biochem. J.* **18**, 891.
—— [1926]. *Ergebn. Physiol.* **25**, 1.
Platt, B. S. & Glock, G. E. [1942]. *Biochem. J.* **36**, *Proc.* xviii.
Rapoport, S. [1940]. *J. biol. Chem.* **135**, 403.
Rosenberger, F. [1908*a*]. *Hoppe-Seyl. Z.* **56**, 373.
—— [1908*b*]. *Hoppe-Seyl. Z.* **57**, 464.
—— [1910]. *Hoppe-Seyl. Z.* **64**, 341.
West, E. S. & Petersen, V. L. [1932]. *Biochem. J.* **26**, 1720.
Williams, R. J., Stout, A. K., Mitchell, H. K. & McMahan, J. R. [1941]. *Studies on the Vitamin Content of Tissues*, **1**. University of Texas Publication No. 4137, University of Texas, Austin, Texas.
Winter, L. B. [1934]. *Biochem. J.* **28**, 6.
—— [1940]. *Biochem. J.* **34**, 249.
Woolley, D. W. [1941*a*]. *J. biol. Chem.* **139**, 29.
—— [1941*b*]. *J. biol. Chem.* **140**, 453.
—— [1942]. *J. exp. Med.* **75**, 277.
Young, L. [1934*a*]. *Biochem. J.* **28**, 1428.
—— [1934*b*]. *Biochem. J.* **28**, 1435.

The Determination of Ascorbic Acid in the Presence of Sulphur Dioxide

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The determination of ascorbic acid with aqueous 2:6-dichlorophenolindophenol is not entirely specific for this vitamin unless special techniques for eliminating interfering substances are applied in each case. SO_2 has a particularly strong reducing action on indophenol, and, when used for the preservation of fruit juices and cordials or in the processing of dried fruits and vegetables, should be removed before the ascorbic acid content of these materials can be accurately determined.

Three methods for eliminating free SO_2 have been described: (1) By boiling the extract and distilling off SO_2 [Kirkpatrick, 1941*a*; Downer, 1942]. (2) By passing a stream of O_2-free N_2 through the extract [Mapson, 1942]. (3) By formation of the non-reducing bisulphite compound of SO_2 with acetone [Mapson, 1942]. SO_2 in fruit juices sweetened with glucose or invert sugar forms the bisulphite compound with the hexose. In amounts of SO_2 up to 1300 parts per million (p.p.m.), indophenol titration is not affected unless the sugar concentration falls below 20% [Kirkpatrick, 1941*b*].

The first two methods are lengthy, and only the third would be practicable as a routine method when many samples are to be examined. In an attempt to find a method by which accurate results may be obtained without the necessity of a fixed rate of titration, the effect of adding H_2O_2, which rapidly converts SO_2 quantitatively into non-interfering H_2SO_4, has now been investigated. Initial experiments showed that the addition of an equal volume of 3% H_2O_2 to an ascorbic acid solution acidified with one drop of concentrated HCl had no appreciable effect on the value obtained by indophenol titration, when the dye was added from the burette. The ascorbic acid was thus unaffected by the H_2O_2, and a diminution in titration value was experienced only when Fe or Cu salts were added to the ascorbic acid, and the solution was allowed to stand for some time before titration.

EXPERIMENTAL

In a detailed study of the effect of adding SO_2 and varying quantities of H_2O_2 to ascorbic acid extracts from various sources, the experiments summarized in Table 1 were carried out. The end-point in each case was reached within 1 min.

Solutions and reagents employed

Ascorbic acid solution: 6·25 mg. dissolved in 100 ml. distilled water. For each titration 10 ml. were acidified with one drop of concentrated HCl.

Orange juice solution: 20% (v/v) in distilled water.

Cauliflower leaf: extract made with 2% HPO_3.

Parsley: extract made with 2% HPO_3.

0·05% *aqueous 2:6-dichlorophenolindophenol*: 1 ml. was equivalent to 0·25 mg. ascorbic acid.

Hydrogen peroxide: a 3% solution of B.P. quality.

$KHSO_3$ *solution*: a 0·934% (w/v) solution in distilled water. 1 ml. added to 10 ml. ascorbic acid solution produced a SO_2 concentration of 450 p.p.m.

DISCUSSION

Table 1 shows that H_2O_2 does not appreciably oxidize ascorbic acid, whether the latter is contained in pure aqueous solution (Exp. 2), orange juice (Exp. 8), or HPO_3 extract of vegetables (Exps. 16 and 19), even after standing for 6 min. The use of HPO_3 as extractant has been reported to guard against the danger of oxidation of ascorbic acid by Fe or Cu, and this probably explains why cauliflower and parsley extracts give good values after H_2O_2 addition, in spite of traces of dissolved mineral salts. The addition of more Fe or Cu salt, however, causes slow oxidation (Exp. 21). H_2O_2 has no effect on unreduced indophenol (Exp. 6).

In all experiments the addition of $KHSO_3$ gives rise to very high titration values, but H_2O_2 neutralizes the effect of the SO_2, and the original titration figure is obtained.

In all experiments in which H_2O_2 has been added to ascorbic acid solutions, the end-point with indophenol is a permanent pink. This colour deepens at varying speeds due to reoxidation of the reduced and decolorized indophenol (leuco-compound) to the original coloured form. The rate of reformation of indophenol is very slow with a concentration of 1·5–2% H_2O_2 (Exps. 10 and 11) and is more rapid with 0·075% H_2O_2 (Exp. 14). The reoxidation of reduced indophenol is still more rapidly catalyzed with added Fe and Cu salts (Exp. 21). The diminution in the titration value with lower concentrations of H_2O_2 (e.g. 0·075%) is most probably due to

Table 1. *Experiments showing the effect of H_2O_2 on the indophenol titration of sulphited and unsulphited ascorbic acid extracts*

Exp. no.	Solution used (ml.)	$KHSO_3$ added (ml.)	3% H_2O_2 added (ml.)	Indophenol titration value (ml.)	End-point and remarks (A = pink; permanent 1 min. B = pink; colour deepens very slowly on standing)
	Ascorbic acid				
1	10	—	—	2·5	A
2	10	—	10	2·45	B
3	10	1	—	>4	Strongly reducing
4	10	1	10	2·45	B
5	10	—	10 (stood 6 min.)	2·3	B
6	Nil	—	10 + 10 ml. water and 1 drop conc. HCl	<0·05	A
	Orange juice				
7	10	—	—	3·9	A
8	10	—	10	3·9	B
9	10	1	—	>7	Strongly reducing
10	10	1	20	3·8	B
11	10	1	10	3·8	B
12	10	1	5	3·8	Pink colour deepens slightly more rapidly
13	10	1	1	3·7	Colour deepens more rapidly
14	10	1	0·5	3·6	Colour deepens rapidly
	Cauliflower leaf				
15	10	—	—	1·52	A
16	10	—	10	1·52	B
17	10	1	10	1·52	B
	Parsley				
18	10	—	—	6·65	A
19	10	—	10 (stood 6 min.)	6·6	B
20	10	1	10	6·65	B
21	10	1 + traces $FeCl_3$ and $CuSO_4$	10	6·1	Pink colour deepens very rapidly

reoxidation of reduced indophenol during the titration, and reduction of the reformed indophenol by ascorbic acid still present. A great advantage of the H_2O_2 procedure is that the pink end-point does not fade.

General method for estimation of ascorbic acid in solutions containing sulphite

The acidity of a measured portion of the solution is brought below pH 3·5 by the addition of HCl, if necessary, and a volume of 3% H_2O_2 equal to that of the whole is added. Indophenol is run in from a burette until a permanent pink colour is obtained. The end-point should be reached within 1 min.

SUMMARY

1. A method for the elimination, by means of H_2O_2, of the interference of SO_2 in ascorbic acid titrations is described.

2. Under the conditions used, H_2O_2 has no appreciable effect on ascorbic acid, unless Fe or Cu salts are present.

REFERENCES

Downer, A. W. E. [1942]. *J. Soc. chem. Ind., Lond.*, **61**, 80.
Kirkpatrick, H. F. W. [1941*a*]. *J. Soc. chem. Ind., Lond.*, **60**, 226.
Kirkpatrick, H. F. W. [1941*b*]. *J. Soc. chem. Ind., Lond.*, **60**, 298.
Mapson, L. W. [1942]. *Biochem. J.* **36**, 196.

The Determination of Ascorbic Acid in the Presence of Interfering Substances, Notably Reductones

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Reductone, reductic acid and other compounds formed in hot alkaline sugar solutions reduce 2:6-dichlorophenolindophenol in the same way as does ascorbic acid. In fact Kertesz [1934] suggested a method of determining the ascorbic acid equivalent of indophenol solutions by titrating the gluco-

reductone produced under standard conditions from glucose and NaOH. Shinohara [1938] observed that the reaction of ascorbic acid with iodine was slow when the acidity was greater than that corresponding with N/2 HCl, the end-point being described as indefinite. This fact has now led to a more detailed investigation into the effect of strong acid on the titration of ascorbic acid with indophenol or iodine.

EXPERIMENTAL

Titration with indophenol

When concentrated HCl is added to ascorbic acid solutions, reversible oxidation becomes progressively slower as the HCl concentration increases. When the HCl content reaches 20% (2·5 parts of 36% HCl to 2 parts of solution) or more, the pink colour produced with one drop of indophenol remains visible for 2 min. On dilution of this solution with 5 vol. of water, indophenol is again reduced to the same extent as in non-acidified solution. The end-point is definite and stable.

Exp. 1. *Stability of ascorbic acid in the presence of* 20% HCl. A solution of ascorbic acid was prepared of indophenol equivalent 3·95 ml./10 ml. Portions of 20 ml. were treated with 25 ml. 36% HCl. This mixture gave no titration. At different times 100 ml. water were added to these 20 ml. portions, and the solution was titrated, the indophenol being added from the burette.

Time (hr.)	Indophenol equiv. (ml.)
0	3·95
1	3·95
3	3·95
24	3·6

The acid thus has no effect on the final titration value after dilution, in periods up to 3 hr.

Exp. 2. *Fresh orange juice titrated with and without the addition of concentrated* HCl:

2 ml. orange juice ≡ 2·3 ml. 0·05% indophenol,
2 ml. + 3·0 ml. 36% HCl ≡ 0·4 ml. indophenol.

2·5 ml. 36% HCl suffices for an initial concentration of 20% HCl, but an extra 0·5 ml. was added in order to compensate for dilution of the acid by the aqueous indophenol. The end-point was taken when the pink colour persisted for 20 sec. ('20 sec. end-point'). On dilution with 25 ml. water the titration was continued to 2·3 ml. The ascorbic acid equivalent is therefore considered to be 1·9 ml. indophenol, the residual 0·4 ml. being due to interfering substances.

Exp. 3. *Guava, lucerne and parsley.* Extracts of these, prepared with HPO_3, gave no indophenol titration in the presence of 20% HCl. These substances thus contain no interfering material, and the total titration is due to ascorbic acid. On dilution with water the full titration value is obtained.

Exp. 4. *Glucoreductone.* A solution of this was prepared by the method of Kertesz [1934]. Of this solution 2 ml. were equivalent to 1·8 ml. indophenol (5 sec. end-point). Preliminary tests showed that the mixture (2 ml. solution + 2·5 ml. 36% HCl) reduced indophenol. The titration was carried out in the following manner. As in Exp. 2, an extra 0·5 ml. HCl was first added, and then the indophenol slowly, with shaking. After the addition of each 0·4 ml. indophenol, an extra 0·5 ml. HCl was required. The end-point (persistence of pink colour for 20 sec.) was reached with 1·8 ml. indophenol and 2·5 ml. additional acid. Thus, under the conditions described, glucoreductone reduces indophenol fully in the presence of HCl. The rate of reduction of indophenol was very much retarded.

Exp. 5. *Titration of ascorbic acid in presence of reductone.* An aqueous ascorbic acid solution was prepared, 1 ml. of which was equivalent to 0·8 ml. indophenol (constant end-point):

2 ml. glucoreductone + 1 ml. ascorbic acid
≡ 2·6 ml. indophenol (5 sec. end-point),

2 ml. glucoreductone + 1 ml. ascorbic acid + HCl (3·8 ml. giving initially 20% HCl + 5 additional quantities of 0·5 ml., as in Exp. 4)
≡ 1·8 ml. indophenol (20 sec. end-point).

On dilution of the last solution with 25 ml. water, a renewed titration of 0·8 ml. indophenol (constant end-point) resulted. The titration in the presence of strong acid gives the glucoreductone equivalent. After dilution with water the extra titration gives the ascorbic acid equivalent.

Differentiation between ascorbic acid and glucoreductone. Three separate titrations are involved: (1) At pH *c.* 3 the titre represents the two substances together. (2) In the presence of at least 20% HCl, indophenol titrates only glucoreductone. The difference between the titrations (1) and (2) represents the ascorbic acid present. (3) On dilution of the reaction mixture from (2) with water and continuation of the titration to the final end-point, the exact equivalent of ascorbic acid is reached. This final titration serves as a check on the value found above by difference.

This technique was successfully applied to an examination of biscuits which had been fortified with concentrated orange juice. High vitamin C values were claimed for these biscuits, but it was found that on titration ordinary plain biscuit gave figures of 20–60 mg. apparent ascorbic acid/100 g. These values were obviously due to reductone-like substances extracted by HPO_3. When the biscuit extracts were treated with HCl as described above, no diminution in the titration value was observed. Thus no ascorbic acid was present in either of the samples. The vitamin C from the orange juice concentrate must have been destroyed in the process of baking.

Titration with N/400 *iodine*

The titration of ascorbic acid with iodine is also affected by the addition of concentrated HCl. The addition of the mineral acid to ascorbic acid solutions at first decreases the rate of reversible oxidation. When a concentration of 29% HCl is reached, iodine is no longer reduced, a higher acid concentration being required for this stage to be reached than in the case of indophenol. On dilution with water the total ascorbic acid reducing capacity is restored. These titrations are best carried out by

adding the iodine to the solution in a 50 ml. pear-shaped separating funnel, containing 1 ml. of $CHCl_3$, and then shaking after the addition of each drop. One drop of N/400 iodine in excess produces a definite pink coloration in the $CHCl_3$ layer. Alternatively, the technique of McHenry & Graham [1935] for the titration of coloured extracts can be applied. At the very high acid concentration required, the titration of glucoreductone is no longer accurate. Consequently, iodine is not a suitable reagent for distinguishing between glucoreductone and ascorbic acid.

Estimation of ascorbic acid under various conditions

(*a*) *In the presence of* $Na_2S_2O_3$. Aqueous solutions of $Na_2S_2O_3$ give the full titration value both with indophenol and iodine in the presence of the requisite amount of HCl, although the rate of reduction is greatly diminished. Either reagent may therefore be used to determine ascorbic acid in the presence of $Na_2S_2O_3$.

(*b*) *In fermented products.* A fermented brew known as 'kaffir beer' made from maize or sorghum malt contains a small amount of ascorbic acid [Delf, 1921]. It has been shown that titration of the brew with indophenol must be carried out at pH 1–2 in order to obtain reliable results [Fox & Stone, 1938]. In the presence of excess HCl a remarkable reaction takes place in that the titration with indophenol is increased instead of decreased. With the iodine technique described above the true ascorbic acid content of the beer can be correctly determined, as in the following experiment:

(*a*) With starch as indicator, 5 ml. beer + 1 drop HCl ≡ 1·0 ml. N/400 iodine.

(*b*) 5 ml. beer + 20 ml. HCl (1 ml. chloroform) ≡ 0·85 ml. N/400 iodine. In this case 4 drops of concentrated HCl were added with each drop of indophenol to maintain the concentration of acid.

(*c*) On dilution of the last solution with water a titration of 1·0 ml. N/400 iodine was obtained.

5 ml. beer thus contained ascorbic acid equivalent to 0·15 ml. N/400 iodine, i.e. 0·66 mg. ascorbic acid/100 ml. This value agrees with that obtained by indophenol titration at pH 1·5.

(*c*) *In urine.* The addition of HCl causes a darkening of urine, and the end-point of the reaction with indophenol becomes difficult to see. This can be greatly improved by preparing a comparison flask of urine and acid. When iodine is the reagent, the end-point is readily seen in the pink colour of the $CHCl_3$ layer.

At about pH 3 the combined titration of ascorbic acid and $Na_2S_2O_3$ in urine gives a satisfactory end-point, but the titration of $Na_2S_2O_3$ in the strongly acid medium is not complete. In the presence of this substance the values found for ascorbic acid would, therefore, be too high.

A suggested explanation of the behaviour of ascorbic acid with concentrated HCl

A solution of ascorbic acid is a tautomeric mixture of the dienol- and keto-enol forms. In a weakly acid solution the mobility of the ionizable H atom permits enolization. However, in the presence of an abundance of H ions due to the addition of HCl, depression of the ionization results in the vitamin being converted completely into the keto-form. Both indophenol and iodine react with the enol-form and not at all with the keto-form. For complete titration free tautomerism from the keto- to the enol-form is necessary. Since no titration takes place with strong HCl, it is probable that only the keto-form is present. On dilution with water, enolization is again facilitated, and titration proceeds to completion.

SUMMARY

1. Ascorbic acid is inert to indophenol or iodine in presence of 20% or 29% HCl, respectively, but on dilution with water the usual reaction occurs.

2. Indophenol is reduced in presence of 20% HCl by glucoreductone and other interfering substances.

3. Ascorbic acid determination in the presence of glucoreductone by a differential titration method is described.

4. It is suggested that, in presence of moderately concentrated HCl, ascorbic acid is in the unreducing keto-form.

REFERENCES

Delf, E. M. [1921]. *Publ. S. Afr. Inst. med. Res.* **2**, no. 14, 47.

Fox, F. W. & Stone, W. [1938]. *S. Afr. J. med. Sci.* **3**, 7.

Kertesz, Z. I. [1934]. *J. biol. Chem.* **104**, 483.

McHenry, E. W. & Graham, M. [1935]. *Biochem. J.* **29**, 2013.

Shinohara, K. [1938]. *J. pharm. Soc. Japan*, **58**, 279.

Studies on the Synthesis of Hyoscyamine in *Atropa belladonna* L. and *Datura stramonium* L.

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In previous papers [Cromwell, 1933, 1937], experiments on the biosynthesis of the alkaloids berberine and hyoscyamine in *Berberis darwinii* and *Atropa belladonna* respectively led to the conclusion that these alkaloids are synthesized from products of carbohydrate and protein breakdown.

This work has been continued with the object of throwing further light on the nature of the intermediate reactions leading to the formation of hyoscyamine in *Atropa belladonna* and *Datura stramonium*.

METHODS

Material. Plants of *Atropa belladonna* and *Datura stramonium* were raised from seed and set out in plots or grown in pots.

Preparation of material for estimation of total alkaloid. Leaves were rapidly dried at a temperature of 60° in an oven and ground to fine powder. Samples of root ¼ in. in diameter were detached from the crowns in the case of *Atropa*, washed thoroughly, cut into pieces and dried at 60°. Roots of *Datura* were also washed, and dried at 60°. When completely dry, the roots were ground to fine powder.

Estimation of total alkaloids. Leaf or root powder (5–10 g.) was exhaustively extracted with dry ethanol in a Soxhlet extractor. The extract was transferred to an evaporating basin and the ethanol removed on a water-bath at 60–70°. The resinous residue was extracted with 50 ml. 0·5N H_2SO_4 and subsequently washed twice with 25 ml. portions of acid. The combined extracts and washings were next shaken with 100 ml. of a mixture of 4 vol. of ether and 1 vol. of chloroform, to remove impurities. The acid extract was made alkaline with dilute ammonia and the bases shaken out with three 100 ml. portions of the ether-chloroform mixture. The three extracts were combined and the solvent distilled off. The residue was treated with two portions of 10 ml. ether which were completely removed by evaporation at 80°. Volatile bases and free ammonia were driven off by heating on the water-bath at 100° for 15 min. The residue was dissolved in 25 ml. N/50 H_2SO_4 and the excess titrated with N/50 NaOH with methyl red as indicator, and the results calculated in terms of hyoscyamine. (1 ml. of N/50 acid ≡ 0·005784 g. hyoscyamine.)

Recovery of hyoscyamine as picrate gave proof that this base is chiefly responsible for the titration values obtained. The theoretical amount of aqueous picric acid was added to the alkaloid solution after neutralization, and the crystals (plates) purified by recrystallizing twice from water. The picrate obtained had m.p. 163–164°. (Found: N, 10·89%. Calc. for hyoscyamine picrate, N, 10·81%.) Pure hyoscyamine picrate has m.p. 165°.

The results obtained from four plants of *Atropa belladonna* are given in the following table:

	Wt. of powder (g.)	Titration value (ml. N/50 H_2SO_4)	Titration value (as g. hyoscyamine)	Theoretical wt. of picrate (g.)	Picrate found (g.)	% recovery
(1)	5 (leaf)	3·9	0·022	0·039	0·0242	62
	5 (root)	5·8	0·033	0·059	0·0386	65
(2)	10 (leaf)	8·1	0·046	0·082	0·0597	73
	10 (root)	10·3	0·059	0·105	0·0793	75
(3)	20 (leaf)	16·7	0·096	0·172	0·1446	84
	20 (root)	20·1	0·116	0·207	0·1782	86
(4)	50 (leaf)	43·2	0·249	0·447	0·4120	92
	50 (root)	54·3	0·314	0·562	0·5152	92

RESULTS

Alkaloid and carbohydrate content of Atropa belladonna

(1) *Normal plants (seasonal variation).* Samples of root, stem and leaves were taken from 2-year-old plants. The figures given in Table 1 represent the average content of six plants. Qualitative experiments on carbohydrate distribution are recorded in Table 2.

Table 1. *Seasonal variation of hyoscyamine content of* Atropa belladonna

Month	g. alkaloid/100 g. dry wt. Root	Stem	Leaf
Jan.	0·42	—	—
Feb.	0·40	—	—
Mar.	0·38	—	0·60 (shoots)
Apr.	0·42	—	0·51 (shoots)
May	0·56	—	0·39
June	0·59	0·12	0·45
July	0·62	0·16	0·48
Aug.	0·61	0·14	0·45
Sept.	0·60	0·10	0·43
Oct.	0·61	0·10	0·43
Nov.	0·56	—	—
Dec.	0·52	—	—

(2) *Etiolated plants.* At the beginning of March, before growth commenced, large plant pots were placed over the crowns of plants. Control samples were taken of roots. At the end of May further

Table 2. *Seasonal distribution of carbohydrates*

	Summer	Autumn	Winter
Roots:			
Reducing sugars	+	+	+
Non-reducing sugars	+	+	+
Starch	+ (wood and pith)	+ + +	+ + +
Stems:			
Reducing sugars	+	+	
Non-reducing sugars	+ +	+	
Starch	–	Trace	
Leaves:			
Reducing sugars	+	Trace	
Non-reducing sugars	+ +	+	
Starch	–	Trace	

Tannins were present in leaves, stems and roots in all seasons. Arginine was present in leaves and roots.

examination of roots was made and also of the etiolated shoots which had been formed. The results of this experiment are shown in Tables 3 and 4.

Table 3. *Hyoscyamine content of etiolated plants*

	g. alkaloid/100 g. dry wt.		
	Roots		
Plant	Control	Experimental	Etiolated shoots
A	0·40	1·10	0·43
B	0·37	0·98	0·45
C	0·41	1·20	0·41
D	0·37	0·88	0·45
E	0·45	1·51	0·47
F	0·42	1·52	0·47

Table 4. *Distribution of carbohydrates in etiolated plants*

	Roots	Etiolated shoots
Reducing sugars	–	+
Non-reducing sugars	+	+ +
Starch	+	–

Arginine was present in both roots and shoots.

The translocation of hyoscyamine in Atropa belladonna

The upward and downward movement of alkaloid was studied by two methods, (*a*) examination of the exudate from cut stems and (*b*) grafting experiments.

Exudate from stems. The shoots of 2-year-old plants were cut to within 3 in. of the crown, and stout rubber tubing, provided with a screw clip, immediately slipped over the stumps. Glass tubes were inserted and supported by retort stands. 10 ml. distilled water were run into the tubes and the level marked. After one week had elapsed the exudate from the stumps was collected and estimated for hyoscyamine. 20 ml. of the exudate from each of three plants gave respectively 1·1, 1·3 and 0·9 mg. hyoscyamine.

Grafting experiments. Experiments were carried out using *A. belladonna* roots as stocks and tomato shoots as scions and vice versa. Samples of root were removed from *A. belladonna* stocks before the experiment, and hyoscyamine estimated. After a period of 5 weeks the tomato scions were examined qualitatively and quantitatively for alkaloid. The Vitali test was applied to a water extract of one of twelve scions and gave a strongly positive reaction for hyoscyamine. The remaining scions were dried and estimated quantitatively. The results are shown in Table 5.

Table 5. *Hyoscyamine content of* A. belladonna *stocks and of tomato scions*

		g. alkaloid/100 g. dry wt.	
		Control	Experimental
(*a*) *A. belladonna* stock:	A	0·52	0·51
	B	0·56	0·53
	C	0·52	0·50
	D	0·54	0·51
(*b*) Tomato scions		0·12	—

The grafting of *A. belladonna* on tomato stocks proved unsatisfactory, only two scions out of twelve surviving. The stocks carrying these scions gave a trace of hyoscyamine by the Vitali test. These experiments show that upward movement of alkaloid from the root system of *A. belladonna* takes place freely, while downward movement from the leaves is very restricted.

The influence of the injection of various substances on the production of alkaloid

Solutions of potassium nitrate, ammonium sulphate, asparagine, urea, nitrogenous bases and amino-acids, alone and in association with glucose, were injected into plants of *Atropa* (2 years old) and *Datura*, with the object of observing the effect on the plants and on the production of alkaloid.

Petiolar and stem injections were first carried out but did not prove satisfactory. Injection through the stump of a stem proved the most rapid and reliable method of introducing solutions into *Atropa* plants, and branch injection in the case of *Datura*.

The solution was contained in a litre aspirator bottle placed 3 ft. from the ground and was conveyed to the cut end of the stump through stout rubber tubing provided with a screw clip. To avoid air locks the tubing was filled with solution by opening the screw clip before connexion was made. With good transpiration as much as 400 ml. of solution passed into *Atropa* plants in 3 days. 100 ml. was the average volume which passed into *Datura* plants. Samples of leaves and roots were taken before and after the injection period.

Preliminary injections were carried out with eosin solution to determine the extent and distribution of injected fluid, and it was found that the dye reached the greater part of the root and shoot system of the plants. In these experiments 2% solutions of glucose and 0·25% of all other substances were used and the duration of injection was 7 days. None of the substances injected at these concentrations appeared to injure the plants.

Table 6. *Influence of the injection of various substances on the alkaloid content of the plants*

(Values given represent the mean of three injections on separate plants.)

Solution injected	g. alkaloid/100 g. dry wt. Leaf Control	Leaf Experimental	Root Control	Root Experimental
A. *Atropa belladonna*				
Potassium nitrate	0·37	0·36	0·55	0·56
Do. + glucose	0·45	0·47	0·64	0·63
Ammonium sulphate	0·43	0·47	0·53	0·55
Do. + glucose	0·47	0·49	0·56	0·60
Asparagine	0·43	0·44	0·60	0·62
Do. + glucose	0·42	0·46	0·59	0·62
Glycine	0·39	0·41	0·55	0·58
Do. + glucose	0·46	0·48	0·63	0·66
Arginine	0·45	0·48	0·65	0·67
Do. + glucose	0·44	0·52	0·58	0·61
Lysine	0·45	0·45	0·55	0·58
Do. + glucose	0·41	0·44	0·52	0·54
Proline	0·42	0·45	0·55	0·56
Do. + glucose	0·41	0·45	0·59	0·61
Putrescine (dihydrochloride)	0·44	0·67	0·62	0·65
Do. + glucose	0·44	0·70	0·60	0·67
Cadaverine (dihydrochloride)	0·39	0·40	0·60	0·62
Do. + glucose	0·41	0·41	0·63	0·64
Choline (chloride)	0·40	0·42	0·61	0·62
Do. + glucose	0·44	0·48	0·60	0·64
Betaine (hydrochloride)	0·45	0·45	0·69	0·65
Do. + glucose	0·39	0·41	0·61	0·62
Methylamine (hydrochloride)	0·42	0·43	0·58	0·60
Do. + glucose	0·38	0·42	0·66	0·69
Dimethylamine (hydrochloride)	0·45	0·45	0·62	0·64
Do. + glucose	0·41	0·45	0·64	0·65
Trimethylamine (hydrochloride)	0·43	0·42	0·57	0·58
Do. + glucose	0·49	0·52	0·59	0·59
Hexamethylene tetramine	0·47	0·49	0·72	0·72
Do. + glucose	0·42	0·47	0·58	0·61
Formamol (hexamethylene tetramine-anhydro-methylene citrate)	0·45	0·48	0·55	0·58
Do. + glucose	0·43	0·49	0·62	0·68
Succinimide	0·44	0·43	0·65	0·67
Do. + glucose	0·49	0·49	0·69	0·68
Urea	0·43	0·43	0·58	0·55
Do. + glucose	0·41	0·42	0·61	0·60
B. *Datura stramonium*				
Asparagine	0·31	0·34	0·25	0·26
Do. + glucose	0·32	0·36	0·26	0·29
Putrescine (dihydrochloride)	0·33	0·33	0·22	0·25
Do. + glucose	0·32	0·36	0·22	0·29
Glycine	0·29	0·31	0·27	0·30
Do. + glucose	0·33	0·36	0·25	0·29
Arginine	0·34	0·34	0·29	0·32
Do. + glucose	0·33	0·36	0·30	0·32
Formamol	0·31	0·35	0·28	0·30
Do. + glucose	0·36	0·39	0·27	0·31

The results of the injection experiments are shown in Table 6; an increase of 0·05 g. hyoscyamine/100 g. dry weight is considered significant.

Injections of arginine with glucose, putrescine alone and with glucose, hexamine with glucose and formamol with glucose gave substantial increases in the hyoscyamine content of *Atropa belladonna*. Putrescine with glucose and formamol alone gave the best results with *Datura stramonium*.

Material from a second experiment on the injection of putrescine was used to check the titration values obtained in the first experiment, and to endeavour to show, by isolation as picrate, that increase of hyoscyamine is responsible for the increased titration value after putrescine injection (Table 7).

The theoretical amount of picric acid was added to the combined neutralized solutions from three plants of *Atropa belladonna* to ensure a good recovery of the alkaloid picrate. 10 g. samples of leaf and root powder were extracted in each case.

Volatile base content of normal and etiolated plants of Atropa belladonna

The volatile bases pyridine, *N*-methyl pyrrolidine and *N*-methyl pyrroline are present in the leaves and roots of *Atropa belladonna* and *Datura stramonium*. Unfortunately a reliable method for separating and estimating these bases could not be devised, but it seemed desirable to examine in normal and etiolated plants the content of volatile bases extractable with ether-chloroform mixture. The procedure adopted was similar to that for the estimation of hyoscyamine with the exception that N NaOH was used for the liberation of the bases, which were then distilled into standard acid. The base content was calculated in terms of *N*-methyl pyrrolidine (Table 8).

DISCUSSION

As a preliminary to further investigations on the nature of the intermediates in the synthesis of hyoscyamine, it was considered desirable to obtain more information on the behaviour of the alkaloid in the plant organs throughout the entire year, and also to carry out translocation experiments with the object of locating the tissues in which synthesis takes place.

The results of experiments on the exudate from cut stems and on grafting indicate clearly that hyoscyamine moves in an upward direction from root to shoot system, and will pass into a scion which does not normally contain it. Although

Table 7. *The effect of injection of putrescine on the yield of hyoscyamine*

	Leaf		Root	
	Control	Experimental	Control	Experimental
(1) Putrescine (dihydrochloride):				
Combined titration value of 3 plants (g. hyoscyamine)	0·123	0·180	0·166	0·184
Theoretical weight of hyoscyamine picrate (g.)	0·220	0·322	0·297	0·329
Weight of picrate found (g.)	0·1871	0·2706	0·2583	0·2896
(2) Putrescine (dihydrochloride) + glucose:				
Combined titration value of 3 plants (g. hyoscyamine)	0·139	0·203	0·155	0·188
Theoretical weight of hyoscyamine picrate (g.)	0·249	0·363	0·277	0·336
Weight of picrate found (g.)	0·2138	0·3195	0·2383	0·2862

The picrates obtained had m.p. 163–164° (N content of sample 10·79%).

these experiments do not prove that the root system is the locus of synthesis, the evidence is strongly in favour of this view. In plants growing under normal conditions it is therefore probable that the alkaloid is formed principally in the root

Table 8. *Volatile base content of normal and etiolated plants*

	Content of volatile base calculated as g. *N*-methyl pyrrolidine/100 g. dry wt.	
(*a*) Normal plants		
	Leaves	Roots
Plant A	0·07	0·04
B	0·09	0·06
C	0·07	0·05
(*b*) Etiolated plants		
	Shoots	Roots
Plant D	0·12	0·09
E	0·14	0·13
F	0·18	0·10

and moves upwards through the vessels into the stems, leaves, fruits and seeds. It would appear that downward movement is very restricted. The work of Dawson [1941] on the tobacco plant similarly leads to the conclusion that nicotine is synthesized mainly in the root and that it moves from the root system via the xylem to the leaves. Shmuck, Smirnow & Ilyin [1941] also conclude from their grafting experiments that the synthesis of nicotine by *Nicotiana* is intimately connected with the root system and the stem. Kerkis & Pigulevskaya [1941] found atropine in all parts of tomato scions grafted on *Datura stramonium*.

The leaves of *Atropa* plants growing in full sun generally give a higher yield of alkaloid than plants growing in shade, and it is possible that the greater transpiration occurring in such plants would tend to draw up more alkaloid from the root system. Sievers [1921], working with *Datura* spp., found that removal of flower buds brought about an increase in alkaloid content of leaves. This finding may be interpreted on the assumption that alkaloid which would normally reach the fruits and seeds would be diverted to the leaves.

The seasonal distribution of alkaloid in roots, stems and leaves justifies, in the main, conclusions drawn from translocation experiments. For leaf tissues a minimum occurred in May (sufficient fully developed leaf material was not available before May), and a maximum in July, after which there was a slight fall until October, when the plants were killed by frost. The low concentration in the stems and the comparatively high concentration in the leaves during autumn show that little downward movement had taken place and therefore, on the subsequent death of leaves and stems, a considerable amount of alkaloid is lost to the plant. The peak of concentration is coincident with full maturity of the plant.

In normal seasons buds develop on the crowns during late autumn and remain dormant throughout the winter. Mild winters, however, may induce considerable growth of these buds, which may assume the form of shoots in February.

Analysis of entire shoots made in early March reveals a substantially high alkaloid content, but leaves detached in May from strongly growing shoots give yields which are low for leaf tissues. In the root a minimum occurs in March and a maximum in July, and from July until November the concentration is well maintained. From November to March an appreciable loss takes place which may be correlated with the development of buds and shoots at this period. From conclusions reached regarding translocation it would appear that loss of alkaloid takes place from root to shoot system. Later work, however, has shown that synthesis may take place to some extent in shoots developing in absence of light, and this was previously found

to be true for leaves from which light was excluded [Cromwell, 1937].

The distribution of carbohydrates follows the course usual for the majority of perennial plants. Starch is the principal carbohydrate stored in the roots during winter and is mobilized in the spring. During the summer months a trace of starch is present in the leaves, whereas mobile sugars are abundant in both leaves and stems. The high concentration of alkaloid found in the roots of plants from which light had been excluded during initial development is particularly significant. Non-reducing sugars and arginine also are present in roots and etiolated shoots, and therefore conditions for synthesis would be optimum, on the assumption that products of carbohydrate breakdown and amino-acids are the essential raw materials. On the other hand, it could be argued that the high content of alkaloid in the roots of these plants is the result of little translocation having taken place into the poorly developed etiolated shoots. Subsequent work has shown, moreover, that etiolated shoots from plants receiving similar treatment are able to synthesize alkaloid to a small extent. The yield of volatile bases is also stimulated by subjecting the plants to continued darkness for 3 months at the beginning of the growing season; hence it may also be assumed that conditions leading to accumulation of amino-acids and carbohydrate derivatives favour synthesis of these bases.

In the injection experiments an increase in alkaloid content of 0·05% over that of the controls is considered significant, although, if limiting factors are operating, it would not necessarily follow that values below this figure rule out the possibility that the substances in question act as intermediates in alkaloid synthesis. It is to be noted that, after injection, the increase of alkaloid in the leaves is usually more marked than in roots, but if synthesis is taking place in the root and the alkaloid moves to the leaves large variations in the root would not be expected. In many instances the stimulating effect of glucose is apparent and is probably due to the provision on breakdown of a ready source of energy and also of intermediates for hyoscyamine production.

Before discussion of the effects of specific substances on injection, it will be convenient to refer to laboratory syntheses of derivatives and near relatives of hyoscyamine. Robinson [1917] synthesized tropinone from succindialdehyde, methylamine and acetone-dicarboxylic acid, highly reactive substances which might occur in the plant. Robinson considers that succindialdehyde and methylamine may arise from the interaction of formaldehyde and ornithine, and acetone-dicarboxylic acid from citric acid. Schöpf & Lehmann [1935] showed that pH affected the yield of tropinone, and obtained at pH 7 a yield of 73% and at pH 5 90%. Schöpf [1937] synthesized teloidine from mesotartaric dialdehyde, methylamine and acetone-dicarboxylic acid.

Of the various amines and amino-acids injected into plants of *Atropa belladonna* and *Datura stramonium*, putrescine, arginine, hexamine and formamol are responsible for the greatest increase in the yield of alkaloid. The effect of putrescine, alone and in association with glucose, is especially marked and suggests that this diamine is an intermediate in the synthesis of hyoscyamine. Arginine injected in association with glucose likewise increases the content of alkaloid substantially. It is therefore inferred that arginine occupies a key position in the synthesis of hyoscyamine as the precursor of putrescine. In processes of bacterial putrefaction, putrescine is generally regarded as arising from arginine via ornithine, and in this connexion Gale [1940] has shown that a mixture of washed suspensions of *Streptococcus faecalis* Sargent and *Escherichia coli* growing in symbiosis produces putrescine from arginine by way of ornithine. The subsequent fate of putrescine is the subject of a later paper in which further experiments will be discussed.

Hexamine and formamol in acid solution both give rise to formaldehyde and ammonium salts, formamol in addition yielding citrate, and hexamine, on reduction, methylamine. These compounds are potential sources of probable intermediates in the synthesis of alkaloids of the tropane group and their effect on injection bears out this conclusion. None of the remaining substances injected gives a significant increase of alkaloid content. Urea appears to inhibit the production of alkaloid, and this observation was also previously made in experiments on urea feeding [Cromwell, 1937]. It is considered that accumulation of urea in the tissues may influence adversely the formation of ornithine from arginine, thus inhibiting the production of putrescine.

Methylamine, dimethylamine, trimethylamine and choline are each responsible for stimulation of alkaloid production when injected with glucose. Ackermann & Schütze [1910, 1911] found methylamine and trimethylamine as the result of bacterial action on choline, and if methylamine is an intermediate in hyoscyamine synthesis it may be derived from choline, which is known to be present in the root of *Atropa belladonna* [Kunz, 1885]. Glycine, however, may provide methylamine on decarboxylation, and this amino-acid also causes a slight increase of alkaloid on injection. Lysine and proline both show a stimulating effect. Ammonium sulphate and asparagine give a positive result which may be due to their indirect effect on amino-acid production. Cadaverine, betaine, succinimide and potassium nitrate are virtually without effect.

SUMMARY

1. The seasonal distribution of hyoscyamine in the leaves, stems and roots of *Atropa belladonna* has been investigated. The maximum concentration in leaves, stems and roots occurs in July, the minimum in leaves during the early part of May and in roots in March. Little downward movement takes place from the leaves in autumn.

2. Translocation experiments lead to the view that the root system is the principal seat of alkaloid synthesis. The alkaloid moves as such from the root system to stems, leaves, fruits and seeds.

3. An approximate estimate of the seasonal distribution of carbohydrates has been made in plants growing under normal conditions and in plants deprived of light.

4. The effects of injection of various amines and amino-acids have been studied. Putrescine, arginine, hexamine and formamol stimulate alkaloid synthesis considerably. Putrescine is regarded as an intermediate in the synthesis of hyoscyamine.

5. Plants sprouting in darkness give a high yield of alkaloid and volatile bases in both roots and etiolated shoots.

6. The significance of these results is discussed.

REFERENCES

Ackermann, D. & Schütze, H. [1910]. *Biol. Zbl.* **24**, 210.
—— —— [1911]. *Arch. Hyg., Berl.*, **73**, 145.
Cromwell, B. T. [1933]. *Biochem. J.* **27**, 860.
—— [1937]. *Biochem. J.* **31**, 551.
Dawson, R. F. [1941]. *Science*, **94**, 396.
Gale, E. F. [1940]. *Biochem. J.* **34**, 853.
Kerkis, J. J. & Pigulevskaya, N. N. [1941]. *C.R. Acad. Sci. U.R.S.S.* **32**, 505.
Kunz, H. [1885]. *Arch. Pharm.* **223**, 701.
Robinson, R. [1917]. *J. chem. Soc.* **111**, 762, 876.
Schöpf, C. [1937]. *Angew. Chem.* **50**, 779, 797.
—— & Lehmann, G. [1935]. *Liebigs Ann.* **518**, 1.
Shmuck, A., Smirnow, A. & Ilyin, G. [1941]. *C.R. Acad. Sci. U.R.S.S.* **32**, 365.
Sievers, A. F. [1921]. *J. Amer. pharm. Ass.* **10**, 674.

The Role of Putrescine in the Synthesis of Hyoscyamine

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When putrescine (tetramethylenediamine) is introduced directly by injection into plants of *Atropa belladonna* and *Datura stramonium*, a considerable increase in the content of hyoscyamine has been found to occur. This result has been interpreted as an indication that putrescine is an intermediate in the synthesis of hyoscyamine [Cromwell, 1943]. If this interpretation is correct it becomes of interest to attempt to discover the manner in which putrescine provides its contribution to the alkaloid molecule. If one bears in mind the use of succindialdehyde in the synthesis of tropinone by Robinson [1917] and Schöpf & Lehmann [1935], the most probable conclusion would be that putrescine first gives rise to succindialdehyde as the result of oxidation. Alternatively, putrescine may undergo partial oxidation with the formation of an aminoaldehyde (γ-aminobutyraldehyde).

The occurrence of a diamine-oxidase system in animal tissues has been reported by Best & McHenry [1930], Zeller [1938, 1940], Zeller, Birkhauser, Mislin & Wenk [1939], and in bacteria by Gale [1942]. Zeller [1938] showed that this enzyme brings about deamination of histamine, putrescine, cadaverine and agmatine, and Gale [1942], working with washed suspensions of *Ps. pyocyanea*, found that at pH 7·5 the oxidation of putrescine, cadaverine and agmatine proceeds to completion. Zeller [1940] reported inhibition of the diamine-oxidase system by cyanide, semicarbazide, choline, monoamines, and over-optimal concentrations of substrate. It was thought that an enzyme bringing about oxidation of putrescine might exist in the tissues of *Atropa*, and accordingly an attempt was made to prepare an active extract from this plant.

EXPERIMENTAL

(1) *Preparation of crude extract from* Atropa *roots, leaves and etiolated shoots.* Freshly gathered material (500 g.) was washed and finely minced with silver sand in a mincing machine. The pulp was transferred to a muslin bag and pressed out in a strong hand-press. Sufficient glycerol was added to the press-juice to make 5% and the press-cake treated with the glycerol solution for ½ hr. The extract was again filtered through muslin and the residue pressed out. The combined press-juice and filtrate was centrifuged

for 15 min. at 2000 r.p.m., the supernatant solution poured off, dialyzed for 12 hr. at 2° and brought to the required pH (7·2 or 6·8).

(2) *Determination of the deaminating activity of extracts.* A preliminary examination of the deaminating activity was made by use of the colorimetric method described by Zeller [1940], who employed potassium indigo disulphonate as an indicator in the diamine-oxidase system, and found that the amount of dye decolorized was proportional to the amount of diamine oxidized.

25 ml. of the enzyme extract of roots were placed in each of five 200 ml. conical flasks (*a*, *b*, *c*, *d*, *e*), and 10 ml. M/15 phosphate buffer (pH 7·2), 2 ml. toluene and 1 ml. 0·05% solution of the dye were all added. Flasks *a*, *b*, *c* each received 5 mg. putrescine dihydrochloride. Flask *c* was boiled for 30 sec. before addition of reagents; *d* and *e* served as controls.

All flasks were closed with rubber stoppers, placed in the incubator at 25°, and shaken periodically. After 5 hr. incubation, decolorization was almost complete in flasks *a* and *b*, but had also progressed in the controls to some extent. No decolorization took place in *c*. This experiment gave some indication of the activity of a diamine-oxidase system in the extract. As a manometric determination of enzyme activity was impracticable, ammonia was determined after incubation for 24 hr. at 25° by aeration followed by titration and Nesslerization.

25 ml. of the extract (pH 7·2 or 6·8) were pipetted into each of seven 200 ml. conical flasks *a*, *b*, *c*, *d*, *e*, *f* and *g*. 10 ml. M/15 phosphate buffer of the required pH were added, followed by 2 ml. toluene. Flasks *a*, *b* and *c* each received 2 ml. of a solution of putrescine dihydrochloride, the equivalent of 5 mg. Flask *d*, which had been boiled for 30 sec., received 5 mg. putrescine dihydrochloride. Flasks *e* and *f* were used as controls and *g* received 5 mg. putrescine dihydrochloride and 2 ml. M/50 KCN. Oxygen was introduced into the flasks in alternate experiments, the flasks closed with rubber stoppers and incubated at 25° for 24 hr. After incubation, *c* was examined for the presence of bacteria, and prior to aeration 5 mg. putrescine dihydrochloride were introduced into the controls. 5 ml. saturated

Table 1. *Deaminating activity of crude extracts of* Atropa

		Ammonia (mg.) and aldehyde (+ etc.) present after incubation of flask					
		a	*b*	*d*	*e*	*f*	*g*
		(*a*) Root extract					
(1) Oxygen, pH 7·2	(Ammonia)	3·02	3·01	2·02	1·97	1·99	1·93
	(Aldehyde)	+ +	+ +	+	+	+	+
(2) Air, pH 7·2		2·62	2·79	2·17	1·95	1·96	2·07
		+ +	+ +	+	+	+	+
(3) Oxygen, pH 7·2		3·09	3·11	2·19	2·07	2·10	2·05
		+ +	+ +	+	+	+	+
(4) Air, pH 7·2		2·24	2·25	2·04	1·85	1·87	1·82
		+ +	+ +	+	+	+	+
(5) Oxygen, pH 6·8		2·24	2·36	2·10	1·98	1·97	1·98
		+	+ +	+	+	+	+
(6) Air, pH 6·8		2·27	2·23	2·22	2·09	2·05	2·04
		+ +	+	+	+	+	+
		(*b*) Extract from etiolated shoots					
(1) Air, pH 7·2		3·38	3·30	3·14	3·13	3·09	3·09
		+ +	+	+	+	+	+
(2) Oxygen, pH 7·2		3·26	3·21	3·04	2·97	2·96	2·99
		+ +	+ +	+	+	+	+
(3) Air, pH 7·2		3·11	3·13	3·04	3·04	2·99	2·97
		+	+	+	+	+	+
(4) Oxygen, pH 7·2		3·14	3·11	2·91	2·86	2·88	2·89
		+ +	+	+	+	+	+
		(*c*) Extract from leaves					
(1) Oxygen, pH 7·2		2·18	2·14	2·16	2·09	2·13	2·09
		+ +	+	+	+	+	+
(2) Air, pH 7·2		2·02	1·92	2·02	1·97	1·93	1·95
		+	+	+	+	+	+
(3) Oxygen, pH 7·2		2·30	2·26	2·31	2·22	2·21	2·21
		+	+	+	+	+	+
(4) Air, pH 7·2		2·19	2·22	2·24	2·17	2·19	2·14
		+	+	+	+	+	+

K_2CO_3 solution were run into each flask and aeration carried out for 40 min. at the temperature of the boiling water-bath. Approximately equal volumes of air were drawn through each of the flasks by means of aspirators, and the ammonia aerated into 25 ml. N/100 acid and back-titrated with N/100 alkali from a micro-burette. After titration the value was checked by the use of Nessler's solution.

Aldehyde production in a series of parallel experiments was gauged by the use of 2 : 4-dinitrophenylhydrazine in 2N HCl. After removal of protein by 10% $CCl_3.COOH$, 5 ml. of a saturated solution of 2 : 4-dinitrophenylhydrazine in 2N HCl were added and the tubes allowed to stand for ½ hr. After this time the tubes were shaken and 10 ml. of the solution transferred to a comparator tube. 5 ml. 20% NaOH were now added and the depth of colour compared in control and experimental tubes.

From Table 1 it will be observed that the presence of introduced putrescine results in an increased formation of ammonia and aldehyde in extracts of root and etiolated shoots but not to any extent in leaf extracts. It thus appears that putrescine is deaminated in the tissues of roots and etiolated shoots, with the formation of ammonia and an aldehyde. The activity of the extract is greater at pH 7·2 than at pH 6·8 and greater in oxygen than in air.

Deamination is inhibited by the presence of cyanide and proceeds independently of bacterial activity.

(3) *Examination of leaves and upper stems of* Atropa belladonna *and* Datura stramonium *for putrescine.* If putrescine is an intermediate in the synthesis of hyoscyamine it would be expected that it might be detectable in the tissues in certain circumstances. The probable occurrence of putrescine in tissues of *Datura stramonium* has been reported by Ciamician & Ravenna [1911], and the presence of a diamine in the leaves of *Atropa belladonna* is mentioned by Greenish [1933]. The tetramethyl derivative of putrescine was isolated in 1907 by E. Merck from *Hyoscyamus muticus* [Henry, 1924].

An examination of leaves and upper stems was therefore made, by the method given below, with the object of isolating the diamine.

2 kg. of tissue were finely minced, treated with 1 l. of saturated ether-chloroform water (4 vol. ether : 1 vol. chloroform) and allowed to stand for 2 hr. The pulp was transferred to a muslin bag, pressed out, and the press-cake extracted twice with water (250 ml.). The combined extracts were heated to 90° and the coagulable protein and tissue debris removed by centrifuging. The clear solution was concentrated to 300 ml. under reduced pressure and treated with lead acetate. The precipitate was filtered off and the lead removed from the filtrate with H_2S. The filtrate was further concentrated, made slightly alkaline with NaOH and the alkaloids shaken out with 3 portions of 100 ml. ether-chloroform mixture. After removal of alkaloids, the alkaline solution was gently warmed under reduced pressure to remove dissolved ether and ammonia. Histidine and arginine were precipitated (at pH 7·2 and 12 respectively) with $AgNO_3$, and the lysine fraction with phosphotungstic acid. The lysine precipitate was suspended in water and the bases regenerated with $Ba(OH)_2$. The filtrate was made slightly acid with dilute HCl and evaporated to dryness *in vacuo*. The residue was next made slightly alkaline with Na_2CO_3, extracted with absolute ethanol, filtered, and the bases precipitated by addition of a saturated solution of $HgCl_2$ in ethanol. After filtration, the precipitate was suspended in water, and mercury removed by H_2S. To the filtrate a few drops of dilute HCl were added, the solution concentrated to 2 ml. and excess of absolute ethanol added. The crystals which separated on standing were removed by filtration, dissolved in the minimum amount of water, reprecipitated with absolute ethanol, and finally recrystallized from absolute methanol. Crystals were obtained which melted at 289°. (Putrescine dihydrochloride has m.p. 290°.) (Found: N, 17·1%. Calc. for putrescine dihydrochloride, N, 17·4%.) The yield of putrescine dihydrochloride from leaves and upper stems of *Atropa belladonna* gathered in May and June was nil, from leaves gathered in August 0·01 g., and from *Datura stramonium* gathered in August 0·022 g. On benzoylation and recrystallization from ether, 10 mg. of the dihydrochloride yielded 15 mg. of crystals which melted at 175–176°. (Found: N, 8·82%. Calc. for dibenzoyl putrescine, N, 8·73%.) A mixed m.p. with authentic dibenzoyl putrescine (m.p. 177°) showed no depression.

On the assumption that putrescine was not lost in manipulation, it is concluded from this examination that at certain periods the diamine shows slight accumulation in leaves and upper stems. During the early part of the growing season accumulation may be prevented by the activity of diamine-oxidase.

Roots, leaves and etiolated shoots of *Atropa* were also examined for the presence of methylamine after ammonia had been removed by yellow HgO, but it was not possible to detect this amine with any degree of certainty. Formaldehyde also was absent from these tissues (dimedon test). Plants which had been injected with choline, betaine, hexamine, trimethylamine, dimethylamine and glycine likewise failed to show the presence of methylamine. Ammonia and aldehyde production, however, was intensified in plants injected with glycine, and also in cut shoots which were allowed to take up this amino-acid from dilute solution.

DISCUSSION

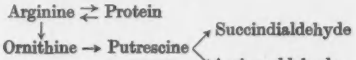
From the results of earlier experiments on *Atropa belladonna* it has been considered that putrescine forms a link in the chain of reactions leading to the synthesis of hyoscyamine and that the most active synthesis takes place in the root system.

The occurrence of an enzyme system which brings about the oxidative deamination of putrescine (and

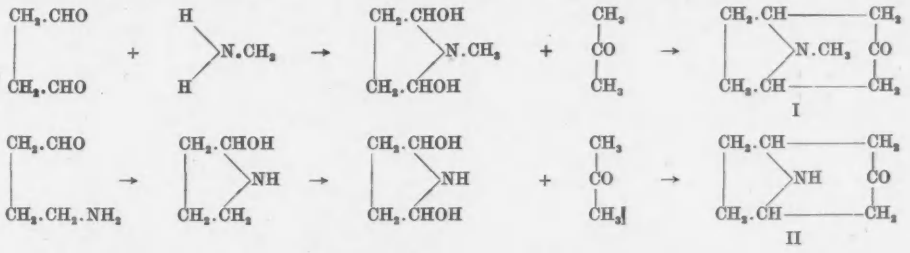
probably cadaverine) in roots and etiolated shoots adds further weight to the above considerations, and gives some degree of biological support to laboratory syntheses of certain members of the tropane group of alkaloids. The optimum pH for diamine-oxidase activity (7·2), however, is higher than that of the cell sap (5·8–6·2), but it does not follow that the pH of the protoplasm of certain cells or tissues must necessarily be the same as that of the cell sap. Fluctuations in pH values may induce the activity of enzymes which are adaptive and thus accumulation of acidic or basic groups may be prevented. For example, Gale [1940] has shown that the amino-acid-decarboxylase activities of bacteria are in some cases strictly adaptive.

Arginine, the precursor of putrescine, either arises as an intermediate in the synthesis of protein or is produced from protein breakdown. In either case it is considered that arginine may be diverted to the production of putrescine.

Partial oxidation of putrescine with the elimination of one molecule of NH_3 would give rise to an amino-aldehyde, and succindialdehyde would be formed as the result of oxidation of both amino groups. Although neither of these aldehydes was isolated, it is presumed that one or both are present when putrescine is subjected to oxidation, in view of the positive reaction with 2 : 4-dinitrophenylhydrazine. The above reactions are represented in the following scheme:

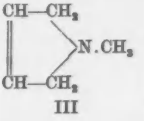


In accordance with the findings and suggestions of Robinson [1917, 1936], succindialdehyde and the amino-aldehyde might contribute to the molecules of tropinone (I) and nor-tropinone (II) according to the scheme outlined below:



Tropinone and nor-tropinone on reduction would yield tropine and nor-tropine, and linkage with tropic acid would then give hyoscyamine and nor-hyoscyamine. If an active acetone derivative (from carbohydrate via citric acid) were not immediately available, it is suggested that the volatile bases N-methyl-pyrrolidine or N-methyl-pyrroline (III) would be produced. Similarly if cadaverine is present (from lysine) and undergoes oxidative deamination to produce glutardialdehyde or an amino-aldehyde, a pyridine ring would result.

The two alkaloids hyoscine and meteloidine, both closely related to hyoscyamine, occur together in *Datura meteloides* and on hydrolysis yield the bases scopine (IV) and teloidine (V) respectively. The occurrence of N-methyl-pyrroline in the leaves of *Atropa belladonna* and *Datura* spp. suggests a possible relationship between this base and scopine and teloidine. If, in N-methyl-pyrroline, the double bond is represented as occurring between carbon atoms 3 and 4 (III), linkage with oxygen would give part of the structure of scopine and subsequent addition of a molecule of water would then yield part of the teloidine molecule. The origin of the double bond might possibly be associated with a dehydrogenase similar to if not identical with succinic dehydrogenase.



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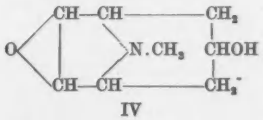

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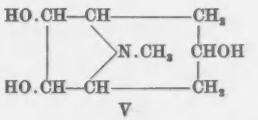

V

As no evidence for the presence of methylamine in the tissues of *Atropa belladonna* and *Datura stramonium* has yet been obtained, the origin of the methyl group attached to the N atom is still obscure. The presence of glyoxylic acid, however, is suspected, and it might possibly be responsible for the methylation of the N atom in the tropane group of alkaloids. Alternatively, an entire methyl group may be transferred from a highly methylated

compound such as betaine or choline [Challenger & Higginbottom, 1935; Challenger, 1942]. The formation of tetramethyl putrescine in *Hyoscyamus muticus* could be explained by assuming either that dimethylamine reacts with succindialdehyde, or that glyoxylic acid (or formaldehyde) brings about direct methylation of putrescine.

SUMMARY

1. An enzyme capable of oxidizing putrescine with the formation of ammonia and an aldehyde has been shown to exist in the tissues of *Atropa belladonna*.

2. The enzyme is located chiefly in the root system, but it also occurs in etiolated shoots.

3. Putrescine has been isolated in small amounts from leaves and upper stems of *Atropa belladonna* and *Datura stramonium*.

4. The possible role of putrescine in the synthesis of hyoscyamine and related alkaloids is discussed.

REFERENCES

Best, C. H. & McHenry, E. W. [1930]. *J. Physiol.* **70**, 349.

Challenger, F. [1942]. *Chem. Ind.* **61**, 397, 413, 456.

—— & Higginbottom, C. [1935]. *Biochem. J.* **29**, 1757.

Ciamician, G. & Ravenna, C. [1911]. *R.C. Accad. Lincei*, **20**, 614. (Quoted from *Chem. Abstr.* 1911, **5**, 3466.)

Cromwell, B. T. [1943]. *Biochem. J.* **37**, 717.

Gale, E. F. [1940]. *Biochem. J.* **34**, 392.

—— [1942]. *Biochem. J.* **36**, 64.

Greenish, H. G. [1933]. *A Text-Book of Pharmacognosy*, 6th ed. London: Churchill.

Henry, T. A. [1924]. *The Plant Alkaloids*, 2nd ed. London: Churchill.

Robinson, R. [1917]. *J. chem. Soc.* **111**, pp. 762, 876.

—— [1936]. *J. chem. Soc.* p. 1079.

Schöpf, C. & Lehmann, G. [1935]. *Liebigs Ann.* **518**, 1.

Zeller, E. A. [1938]. *Helv. chim. Acta*, **21**, 1645.

—— [1940]. *Helv. chim. Acta*, **23**, 1418, 1502.

—— Birkhauser, H., Mislin, H. & Wenk, M. [1939]. *Helv. chim. Acta*, **22**, 1381.

Studies in the Biochemistry of Micro-organisms

72. *GENTISYL ALCOHOL* (2:5-*DIHYDROXYBENZYL ALCOHOL*), A METABOLIC PRODUCT OF *PENICILLIUM PATULUM* BAINIER

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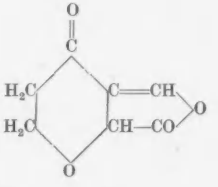
Workers in this laboratory [Birkinshaw, Bracken, Michael & Raistrick, 1943] have recently described the isolation of patulin, $C_7H_6O_4$, believed to be anhydro-3-hydroxymethylene-tetrahydro-γ-pyrone-2-carboxylic acid (I), from cultures of *Penicillium patulum* Bainier grown on Raulin-Thom medium. Patulin is a strong anti-bacterial substance. It completely inhibits the growth of a large series of Gram-positive and Gram-negative bacteria at concentrations of 1 : 33,000 to 1 : 100,000. It is also a powerful anti-fungal agent, since it completely inhibits, at a concentration of 1 : 400,000, the growth of a number of species of *Pythium* which are plant pathogens [Anslow, Raistrick & Smith, 1943].

Birkinshaw *et al.* [1943] recorded that the ethereal mother liquors, from which patulin had been isolated, still contained other metabolic products of *P. patulum*. One of these products, which forms the subject of the present communication, has now been isolated, in yields of 0·6 g./l. of culture filtrate, and identified as *gentisyl alcohol* (2 : 5-*dihydroxybenzyl alcohol*) (II). Gentisyl alcohol has only slight anti-bacterial activity against *Staphylococcus aureus*.



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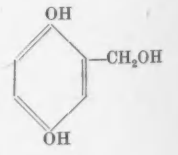

II

It is a curious fact that, so far as we can ascertain, a compound of this simple chemical constitution has not been described previously, although the corresponding aldehyde, gentisic aldehyde, and the corresponding acid, gentisic acid, which is also formed by *P. patulum*, have been known for a long time.

The chemical constitution of the new metabolic product was established as follows. It has the empirical formula, $C_7H_8O_3$. On long continued reduction with Pd-norite-H_2 it is converted into toluquinol. Oxidation of the dimethyl ether of the metabolic product with $KMnO_4$ gives 2 : 5-dimethoxybenzoic acid which on demethylation affords gentisic acid (2 : 5-dihydroxybenzoic acid). Finally, gentisyl alcohol was synthesized by the partial reduction of gentisic aldehyde, the initial quick reduction being stopped after the absorption of 1 mol. of hydrogen. Synthetic gentisyl alcohol and the new mould metabolic product have the same m.p. 100°, and a mixture of the two substances melts at the same temperature.

EXPERIMENTAL

Isolation of the new metabolic product. Cultures of *Penicillium patulum* Bainier, strain Ad 77, on Raulin-Thom solution, were prepared in the usual way. After 14 days' incubation at 24° the bulked culture filtrate from 52 flasks had the following characteristics: total vol. 16·14 l.; residual glucose by polarimeter, 0·74%; pH, 5·0; anti-bacterial activity against *Staphylococcus aureus*, complete inhibition at a dilution of 1 : 160, partial inhibition at 1 : 320. The bulked filtrate was evaporated *in vacuo* to 800 ml. and extracted three times with 800 ml. each time of ethyl acetate. The extract was dried over Na_2SO_4 and the solvent was removed *in vacuo*. The residual oil (58 g.) which was beginning to crystallize was dissolved in ether and passed through a column of alumina as described by Birkinshaw *et al.* [1943]. Two crops of patulin, wt. 19·42 and 0·88 g., were isolated.

The ethereal mother liquors remaining after separation of the patulin were freed from solvent and the residual oil was dissolved in ether (150 ml.) and hot chloroform (1 l.) was added. The crude metabolic product separated in clusters of needles containing a little oil. The product was collected after 4 days and washed with a little ether. Wt. 6·98 g., m.p. 98–99·5°. The mother liquor, after standing in an open flask for a considerable time, deposited a second crop of needles mixed with some oil. Wt. 2·90 g., m.p. 84–96°.

Purification of the crude metabolic product presented some difficulty owing to the tendency of the substance to separate as an oil. It also appears to be somewhat sensitive to heat. No completely satisfactory solvent was found and the method finally adopted was as follows: A portion (4 g.) of the first crop was finely powdered and added to 1 l. of boiling chloroform. The mixture was refluxed for 5 min. and filtered. On cooling, 1·10 g. of almost colourless needles were obtained; m.p. 100° after sintering at 98°. The undissolved portion, 1·97 g., was reworked in subsequent crystallizations.

General properties of the metabolic product. Colourless needles, m.p. 100°, which sublime in a high vacuum at a bath temperature of 70–80°, though not as readily as might be expected of a substituted quinol. (Found: C, 59·87, 60·01; H, 5·89, 6·06; N, nil; OCH_3, nil. $C_7H_8O_3$ requires C, 60·01; H, 5·75%. 2·53 active H atoms in pyridine, 0·41 atoms in anisole. Kuhn-Roth method gives 0·112 mol. acetic acid.)

It is readily soluble in water, ethanol and ether, slightly soluble in benzene and chloroform and insoluble in light petroleum. An aqueous solution has the following properties: neutral to litmus; instantly decolorizes $KMnO_4$; reduces $AgNO_3$ in the cold with mirror formation; gives an evanescent blue colour with aqueous $FeCl_3$, changing almost immediately to yellow; reacts with aqueous iodine with absorption of two atoms of iodine which is slowly lost on titration with thiosulphate; does not react with hydroxylamine even after 24 hr. With 2N NaOH it gives an immediate yellow colour rapidly darkening to strong reddish brown. When heated with 2N H_2SO_4, in which it is readily soluble in the cold, it gives in a few seconds a white amorphous precipitate.

It gives nearly complete inhibition of the growth of *Staphylococcus aureus* at a concentration of 1 : 500, partial inhibition at 1 : 1000 and 1 : 2000 and no inhibition at 1 : 4000.

Catalytic reduction of the metabolic product. Formation of toluquinol. A solution of the metabolic product (0·5 g.) in ethanol (10 ml.) was hydrogenated at ordinary pressure with a palladium-norite catalyst prepared from 0·2 g. of $PdCl_2$ and 0·5 g. of norite. Hydrogenation, which was slow throughout, was discontinued after 8¾ hr. (Uptake of hydrogen, 57·8 ml. (corr.); theor. for uptake of 2H per mol., 80 ml.) After filtering from catalyst the ethanol was removed *in vacuo*. A mass (0·48 g.) of crystals containing some oil remained. This material sublimed readily in a water-pump vacuum at a bath temperature of 110–115°. The sublimate formed large colourless blades, m.p. 127°, setting on cooling and remelting at 127°. Resublimed in a high vacuum the sublimate melted at 128°, alone or in admixture with authentic, sublimed toluquinol, m.p. 128°. (Found: C, 67·39; H, 6·40. Calc. for $C_7H_8O_2$: C, 67·75; H, 6·50%.)

Methylation of the metabolic product. A solution of the metabolic product (2·0 g.) in dry ether (25 ml.) was treated with an excess of ethereal diazomethane for 3 days. On removal of the solvent a reddish oil (2·35 g.) remained, which, on distillation *in vacuo*, gave 1·46 g. of a fairly mobile yellow oil, b.p. 140°/3·3 mm. (Found: OCH_3, 34·1. $C_9H_{12}O_3$ requires, for $2OCH_3$, 36·9%.)

Oxidation of crude dimethoxy derivative. Formation of 2 : 5-dimethoxybenzoic acid and demethylation

to gentisic acid. The above oil (1 g.) was suspended in hot water (50 ml.) containing N KOH (1 ml.) and to it was added a solution of $KMnO_4$ (1·5 g.) in hot water (150 ml.). After ½ hr. heating on the water-bath no $KMnO_4$ remained. The solution was filtered, the filtrate evaporated to 25 ml. and acidified to Congo red with 2N HCl (3 ml.). An emulsion was formed which rapidly crystallized, forming colourless needles, wt. 0·55 g., m.p. 72–76°. After recrystallization from water the oxidation product melted at 75–76°, not depressed on admixture with authentic, synthetic 2 : 5-dimethoxybenzoic acid. (Found: C, 59·30; H, 5·60. Calc. for $C_9H_{10}O_4$: C, 59·33; H, 5·53%.)

The acid (0·13 g.) was heated with HI (sp. gr. 1·7; 3 ml.) in a Zeisel apparatus in a stream of CO_2, the bath temperature being maintained at 130–140° during 1 hr. On cooling, fine brownish needles separated. These were collected and washed with conc. HCl. Wt. 0·07 g., m.p. 199–201°. The crystals gave a colourless crystalline sublimate in a high vacuum, m.p. 204° not depressed on admixture with authentic gentisic acid, m.p. 203°. (Found: C, 54·51; H, 4·09. Calc. for $C_7H_6O_4$: C, 54·55; H, 3·92%.)

Synthesis of gentisyl alcohol (2 : 5-*dihydroxybenzyl alcohol*). *Identity with metabolic product.* Gentisic aldehyde (2 : 5-dihydroxybenzaldehyde) (0·5 g.), prepared from salicylaldehyde by the method of Neubauer & Flatow [1907] and dissolved in ethanol (10 ml.), was hydrogenated at ordinary pressure with a palladium-norite catalyst prepared from 0·2 g. of $PdCl_2$ and 0·5 g. of norite. The uptake of hydrogen was fairly rapid. Hydrogenation was discontinued after 50 min. (Uptake of hydrogen, 82·5 ml. (corr.); theor. for uptake of 1 mol. H_2, 81·2 ml.) After filtering from catalyst the ethanol was removed *in vacuo*. A yellow oil (0·57 g.) remained and began to crystallize overnight. The product was recrystallized from chloroform in the same way as is described above for the purification of the crude metabolic product. After two recrystallizations it was obtained as almost colourless needles, m.p. 99° after softening at 95°. It was finally sublimed in a high vacuum. Colourless crystalline sublimate, m.p. 100°, alone or in admixture with the metabolic product. It also gave the same reactions. (Found: C, 60·09; H, 5·79. Calc. for $C_7H_8O_3$: C, 60·01; H, 5·75%.)

SUMMARY

Gentisyl alcohol, 2 : 5-*dihydroxybenzyl alcohol*, occurs together with patulin as a metabolic product of *Penicillium patulum* Bainier. Its isolation, identification and synthesis are described.

REFERENCES

Anslow, W. K., Raistrick, H. & Smith, G. [1943]. *J. Soc. chem. Ind., Lond.*, **62**, 236.

Birkinshaw, J. H., Bracken, A., Michael, S. E. & Raistrick, H. [1943]. *Lancet*, **2**, 625.

Neubauer, O. & Flatow, L. [1907]. *Hoppe-Seyl. Z.* **52**, 375.

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